

Citation for published version:

Dayalan Naidu, S, Sutherland, C, Zhang, Y, Risco, A, de la Vega, L, Caunt, CJ, Hastie, CJ, Lamont, DJ, Torrente, L, Chowdhry, S, Benjamin, IJ, Keyse, SM, Cuenda, A & Dinkova-Kostova, AT 2016, 'Heat shock factor 1 is a substrate for p38 mitogen-activated Protein Kinases', *Molecular and Cellular Biology*, vol. 36, no. 18, pp. 2403-2417. <https://doi.org/10.1128/MCB.00292-16>

DOI:

[10.1128/MCB.00292-16](https://doi.org/10.1128/MCB.00292-16)

Publication date:

2016

Document Version

Peer reviewed version

[Link to publication](#)

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1 **Heat Shock Factor 1 is a Substrate for p38 Mitogen-Activated**
2 **Protein Kinases**

3
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21 **Running Title:** HSF1 is a substrate for p38 MAPK
22

23 **Keywords:** cellular stress responses, p38 γ , p38 δ , PEITC, phosphorylation
24

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29

30 **Abstract**

31 Heat Shock Factor 1 (HSF1) monitors the structural integrity of the proteome.
32 Phosphorylation at S326 is a hallmark for HSF1 activation, but the identity of the
33 kinase(s) phosphorylating this site has remained elusive. We show that the dietary
34 agent phenethyl isothiocyanate (PEITC) inhibits heat shock protein 90 (Hsp90), the
35 main negative regulator of HSF1, activates p38 MAPK, increases S326
36 phosphorylation, trimerization and nuclear translocation of HSF1, and the
37 transcription of a luciferase reporter as well as the endogenous prototypic HSF1 target
38 Hsp70. *In vitro*, all members of the p38 mitogen-activated protein kinase (MAPK)
39 family rapidly and stoichiometrically catalyze the S326 phosphorylation. The use of
40 stable knockdown cell lines and inhibitors indicated that among the p38 MAPK, p38 γ
41 is the principal isoform responsible for the phosphorylation of HSF1 at S326 in cells.
42 A protease-mass spectrometry approach confirmed S326 phosphorylation, and
43 unexpectedly, revealed that p38 MAPK also catalyze phosphorylation of HSF1 at
44 S303/307, previously known repressive post-translational modifications. Thus, we
45 have identified p38 MAPK as highly efficient catalysts for the phosphorylation of
46 HSF1. Furthermore, our findings suggest that the magnitude and persistence of
47 activation of p38 MAPK are important determinants of the extent and duration of the
48 heat shock response.

49

50 Introduction

51 Heat Shock Factor 1 (HSF1) orchestrates an elaborate transcriptional program which
52 enhances adaptation and survival under conditions of stress. It is activated in response
53 to stresses such as heat shock, hypoxia, heavy metals, reactive oxygen species and
54 changes in pH. In an unstressed system, monomeric HSF1 is bound to its negative
55 regulators, heat shock proteins (Hsp) 40, 70 and 90 (1-3). During stress, HSF1 is
56 released from the complex, and undergoes several activating post-translational
57 modifications that allow it to form a transcriptionally active trimer. In the nucleus,
58 trimeric HSF1 binds to heat shock elements (HSE, comprising the consensus inverted
59 repeat sequences nGAAn) to orchestrate transcription of large networks of
60 cytoprotective genes, including molecular chaperones, DNA damage repair
61 components, and metabolic enzymes (4). Activation of HSF1 plays a vital role in
62 human physiology and ageing, as well as in pathological processes such as
63 cardiovascular disease, neurodegeneration, and cancer.

64 Increased nuclear HSF1 levels correlate with poor prognosis in breast, colon
65 and lung cancer (5, 6). Furthermore, it is becoming increasingly clear that HSF1 is
66 able to support the malignant phenotype by orchestrating a transcriptional program
67 beyond the heat shock response, including energy metabolism (5, 7). In addition,
68 some of the downstream target genes of HSF1 encode proteins involved in global
69 protein translation, such as the RNA-binding protein HuR (8, 9). Santagata *et al.* (10)
70 have reported that inhibition of protein translation in malignant cells reduced the
71 activation of HSF1, providing an insight that a close relationship exists between the
72 translational machinery and the transcriptional program orchestrated by HSF1. These
73 findings raise the possibility of targeting HSF1 by inhibiting the cellular processes
74 that lead to activation of the transcription factor in cancer.

75 The activity of HSF1 is controlled by a wide range of post-translational
76 modifications. Westerheide *et al.* (11) have reported that activation of the deacetylase
77 and longevity factor SIRT1 maintains HSF1 in a deacetylated, DNA-binding
78 competent state, and extends the duration of the heat shock response. Raychaudhuri *et*
79 *al.* (12) discovered that EP300/CREB, a histone acetyltransferase, is responsible for
80 stabilization of HSF1 through acetylation of several of its lysine residues. In addition
81 to acetylation, various phosphorylation modifications cause the transcription factor to
82 become either transcriptionally repressed or activated. Most phosphorylation

83 modifications occur within the regulatory domain (RD) of HSF1 and are inhibitory.
84 Indeed, a recent study has shown that a phosphorylation-deficient HSF1 mutant, in
85 which the 15 known phosphorylation sites within the RD had been disrupted
86 (HSF1 Δ pRD), is a potent transactivator under stress conditions, and has a lower
87 activation threshold than its wild-type counterpart (13). In human HSF1,
88 phosphorylation at S303 [PPS³⁰³PPQS³⁰⁷PRV] after obligatory priming
89 phosphorylation at S307 by the mitogen-activated protein kinase (MAPK) ERK1, is
90 carried out by glycogen synthase kinase (GSK)3, inhibiting the function of the
91 transcription factor (14-17). Similarly, phosphorylation at S121, catalyzed by MAPK-
92 activated protein kinase 2 (MK2), inhibits the transcriptional activity of HSF1 and
93 promotes its binding to Hsp90 (18). In stark contrast, phosphorylation at S326
94 [VDTLSS³²⁶PTAL] activates HSF1, and the mutation of S326 to alanine (S326A)
95 reduces its transcriptional activity by more than 80% (19, 20). To our knowledge, the
96 identity of the kinase(s) phosphorylating this site has not been clearly established.

97 Diets rich in cruciferous vegetables have protective effects against
98 neurodegenerative and cardiovascular disease, and cancer (21). Watercress
99 (*Nasturtium officinale*), a vegetable from this family, is a rich source of the
100 glucosinolate gluconasturtiin. Phenethyl isothiocyanate (PEITC) (**Fig. 1A**) is an
101 isothiocyanate (ITC) that forms during plant tissue injury from gluconasturtiin,
102 through the catalytic action of myrosinase [E.C. 3.2.1.147], a β -thioglucosidase (22,
103 23). PEITC is currently in clinical trials for prevention of lung cancer and for
104 depletion of oral cells expressing mutant p53 in people who smoke
105 (ClinicalTrials.gov). Due to the presence of the electrophilic isothiocyanate group,
106 which reacts readily with sulfhydryl groups, PEITC is an activator of transcription
107 factor nuclear factor-erythroid 2 p45-related factor 2 (NRF2), a master transcriptional
108 regulator of antioxidant, anti-inflammatory, and drug-metabolizing enzymes. Global
109 gene expression profiling of murine liver has revealed that, in addition to classical
110 NRF2-dependent genes, a single dose (40 mg/kg) of orally-administered PEITC
111 induces transcription of heat shock proteins (24), but how this occurs is not known.
112 Interestingly, PEITC has been reported to activate signal transduction cascades,
113 including protein kinases (25). Here we show that PEITC activates p38 MAPK,
114 causes phosphorylation of HSF1 at S326, and transcriptionally activates HSF1. We

115 further identify the family of p38 MAPK as highly efficient catalysts of the
116 phosphorylation of HSF1.

117

118 **Results**

119 **Cysteine-reactive PEITC induces the heat shock response.** We have previously
120 reported that structurally diverse NRF2 activators, all of which react with sulfhydryl
121 groups, induce the heat shock response, and demonstrated the essential requirement
122 for HSF1 (26). The isothiocyanates represent a prominent class of NRF2 activators,
123 which have shown chemoprotective effects in numerous animal models of chronic
124 disease; some have been and/or currently are in clinical trials (27-29). We therefore
125 examined the potential heat shock response-inducer activity of three representative
126 isothiocyanates: allyl- (AITC), benzyl- (BITC), and phenethyl isothiocyanate
127 (PEITC) (**Fig. 1A**) in the human breast cancer cell line MDA-MB-231, using Hsp70
128 as a prototypic heat shock protein. When cells were exposed for 24 h to the aromatic
129 isothiocyanates BITC or PEITC at a concentration of 10 μ M, the levels of Hsp70
130 increased by ~12- and ~10-fold, respectively, whereas the levels of Hsp70 remained
131 unchanged upon exposure to 10 μ M of the aliphatic isothiocyanate AITC (**Fig. 1B**).
132 PEITC is in clinical trials for prevention of lung cancer and for depletion of oral cells
133 expressing mutant p53 (ClinicalTrials.gov). We therefore focused our subsequent
134 studies on this isothiocyanate. Experiments in mouse embryonic fibroblasts (MEFs)
135 confirmed the requirement for HSF1 for the induction of Hsp70 by PEITC. In wild-
136 type MEFs, exposure to 7.5- or 10 μ M PEITC for 24 h caused an upregulation of
137 Hsp70 by ~2- and ~3.2-fold, respectively, whereas the levels of this heat shock
138 protein remained unchanged in their HSF1-deficient counterparts (**Fig. 1C**).
139 Consistent with the increase in the protein levels of Hsp70, the mRNA levels for
140 *hsp70* were upregulated by 4.1- and 3.5-fold after exposure of wild-type MEF cells
141 to 10 μ M PEITC for 8- or 16 h, respectively (**Fig. 1D**).

142 Nuclear-cytoplasmic separation experiments conducted in MDA-MB-231
143 cells showed that PEITC caused nuclear translocation of HSF1 (**Fig. 1E**). Thus, in
144 vehicle-treated cells, HSF1 was present in both the cytoplasmic and nuclear fractions.
145 In sharp contrast, in the cytoplasmic fraction of cells treated with PEITC for 3 h, there
146 was no detectable HSF1, and essentially all HSF1 was in the nuclear fraction.
147 Furthermore, the presence of monomeric, dimeric and trimeric HSF1 species was

148 readily detectable in the nuclear fraction of PEITC-treated cells. Collectively, these
149 experiments show that upon PEITC treatment, HSF1 undergoes nuclear translocation
150 and trimerization. Notably, the gel electrophoretic mobility of monomeric HSF1 in
151 the nuclear fraction of PEITC-treated cells was slower than in their vehicle-treated
152 counterparts (**Fig. 1E**), indicative of occurrence of post-translational modifications.

153 Trimerization is required for the transcriptional activity of HSF1 (30-33). To
154 test whether the HSF1 trimers that form upon treatment with PEITC are able to
155 enhance transcription through heat shock elements (HSEs), we used the cervical
156 cancer HeLa HSE-luciferase reporter cell line (HeLa-HSE-luc) stably transfected with
157 the *HSP70.1* promoter fused to the luciferase gene (34). Remarkably, PEITC led to a
158 dramatic dose- and time-dependent induction of the reporter, with a maximal increase
159 of more than 1000-fold (**Fig. 1F**). Together, these results demonstrate that PEITC is a
160 potent and robust inducer of the heat shock response.

161

162 **PEITC inhibits Hsp90.** Activation of HSF1 requires release from its negative
163 regulators; indeed, inhibition of Hsp90, the main negative regulator of HSF1, often
164 leads to induction of the heat shock response (35). To test whether PEITC inhibits the
165 function of Hsp90, we evaluated the stability of two well-established Hsp90 client
166 oncoproteins, the tyrosine kinase HER2 and the serine/threonine kinase RAF1, both
167 of which bind strongly to Hsp90 (36). After treatment with PEITC, the levels of
168 HER2 and RAF1 were decreased by ~60- and ~35%, respectively, consistent with
169 Hsp90 inhibition (**Fig. 2A**). Treatment with 10 μ M PEITC did not lead to any changes
170 in the transcription of either of these genes, as quantified by real-time PCR.
171 Interestingly however, the 20- μ M PEITC treatment led to a significant ($p = 0.002$, n
172 = 3) 45% decrease in the mRNA levels for HER2, although the mRNA levels for
173 RAF1 were not affected (not shown). The reason for this decrease in HER2
174 expression, and its potential contribution to the decreased HER2 protein levels at the
175 high concentration of PEITC is presently unknown. In contrast to geldanamycin (GA),
176 an Hsp90 inhibitor that competes with ATP, but similarly to celastrol (CL) and
177 sulfoxythiocarbamate alkyne (STCA), which inhibit Hsp90 by modifying its cysteine
178 residues without interfering with ATP binding (37), PEITC did not prevent the ability
179 of the chaperone to bind ATP (**Fig. 2B**). These results support the notion that PEITC,
180 by virtue of its cysteine reactivity inhibits Hsp90, and further suggest that, by
181 inhibiting Hsp90, the isothiocyanate may trigger the release of HSF1. Indeed,

182 immunoprecipitation experiments showed that the amount of HSF1 bound to Hsp90 is
183 greatly reduced upon exposure to PEITC (**Fig. 2C**).

184

185 **PEITC causes phosphorylation of HSF1 at S326.** In addition to release from Hsp90,
186 full transcriptional activation of HSF1 requires its phosphorylation at S326 (19, 20).
187 The shift in gel electrophoretic mobility of the monomeric form of HSF1 in nuclear
188 fractions of PEITC-treated cells (**Fig. 1D**) indicated the occurrence of post-
189 translational modifications. Given the dramatic activation of the HSE-luciferase
190 reporter by PEITC (**Fig. 1F**), we tested the possibility that exposure to PEITC was
191 causing HSF1 phosphorylation at S326. The use of a pS326-phosphospecific antibody
192 revealed a time- and dose-dependent increase in pS326 HSF1 and an upregulation of
193 Hsp70 upon exposure to PEITC (**Fig. 3A**). Notably and in full agreement with the
194 shift in electrophoretic mobility of HSF1 in nuclear fractions of PEITC-treated cells
195 (**Fig. 1D**) as well as in the immunoprecipitation experiment (**Fig. 2C**), in this
196 experiment the migration of HSF1 was slower in lysates from cells treated with 20
197 μ M PEITC than from vehicle- or 10 μ M PEITC-treated cells (**Fig. 3A**). Surprisingly
198 however, although HSF1 S326 phosphorylation was more extensive upon treatment
199 with the higher concentration (20 μ M) of PEITC, induction of Hsp70 appeared to be
200 greater at the lower concentration (10 μ M) of the isothiocyanate. Nuclear-cytoplasmic
201 separation further confirmed the accumulation of pS326 HSF1 in both cytoplasmic
202 and nuclear fractions (**Fig. 3B**). The results from this shorter time-course experiment
203 also indicated that HSF1 phosphorylation occurred in the cytoplasm and preceded the
204 nuclear translocation of the transcription factor.

205

206 **PEITC activates p38 MAPK.** S326 of HSF1 represents a proline-directed
207 phosphorylation site. Only a stringent subset of kinases, known as CMGC kinases are
208 able to phosphorylate proline-directed sites (38). As PEITC has been reported to
209 activate p38 mitogen-activated protein kinases (MAPK) (25), a proline-directed
210 family of kinases, we next examined their status in MDA-MB-231 cells. A dose-
211 dependent phosphorylation of p38 MAPK was readily detectable, and increased by
212 ~30- and ~90-fold, after treatment with 10- and 20 μ M of PEITC, respectively (**Fig.**
213 **4A**). Importantly, the levels of p38 MAPK were unchanged, showing that PEITC did
214 not cause any alterations in protein expression or stability of the kinases. In contrast to

215 the activation of p38 MAPK, exposure to PEITC decreased the phosphorylation of the
216 ribosomal subunit S6 at S235/236 (Fig. 4B), indicating inhibition of the mechanistic
217 target of rapamycin (mTOR), a kinase that had been previously implicated in the
218 phosphorylation of HSF1 at S326 (20). Moreover, the dose-dependent PEITC-
219 mediated phosphorylation of p38 MAPK correlates well with the extent of
220 phosphorylation of HSF1 at S326, which increased by 30- and 55-fold, after treatment
221 with 10- and 20 μ M PEITC, respectively (Fig. 4A).

222 It has been reported that the c-Jun N-terminal kinases (JNK) phosphorylate
223 and activate HSF1 (39). We therefore next tested the effect of JNK-In-8, a JNK-
224 selective inhibitor (40) and BIRB0796, a p38 MAPK inhibitor (41), on the ability of
225 PEITC to induce phosphorylation of HSF1 at S326. Both inhibitors decreased the
226 PEITC-mediated phosphorylation of HSF1 at S326 (Fig. 4C). However, a time course
227 experiment further revealed that, although both p38 and JNK1/2 were activated by
228 exposure to 20 μ M PEITC, the activation of JNK1/2 displayed a delayed kinetics in
229 comparison with the kinetics of activation of p38 MAPK (Fig. 4D).

230 Recently, it was reported that HSF1 physically interacts and is phosphorylated
231 at S326 by MAPKK (also known as MEK) (42). We therefore next examined the
232 effect of inhibiting MEK on HSF1 S326 phosphorylation using the highly selective
233 MEK1/2 inhibitor 1,4-diamino-2,3-dicyano-1, 4-bis[2-aminophenylthio]butadiene
234 (U0126) (43). In agreement with the published report (42), we were able to detect
235 reduced levels of phosphorylation of HSF1 at S326 in lysates of heat-shocked cells
236 that had been pre-treated for 24 h with U0126 (Fig. 5A). However, the MEK inhibitor
237 had no effect on the phosphorylation of HSF1 at S326 in heat-shocked cells that had
238 been pre-treated with U0126 for 1 h (Fig. 5B). Overall, these findings raise the
239 possibility that p38 MAPK could represent one group of the long-sought catalysts for
240 the phosphorylation of this key residue.

241
242 **p38 MAPK phosphorylate HSF1 at S326 *in vitro*.** Next, we used recombinant HSF1
243 to test the ability of purified recombinant p38 MAPK isoforms to phosphorylate
244 HSF1 *in vitro*. HSF1 was expressed as a His-tagged fusion protein in *Escherichia coli*.
245 Purified His-HSF1 migrated as a major band during NuPAGE® at the expected
246 molecular weight, and showed a tendency to form spontaneously dimeric and trimeric
247 species (Fig. 6A). The four p38 MAPK isoforms (α , β , γ and δ) were expressed

248 individually in *Escherichia coli* from human cDNAs as inactive GST-fusion proteins,
249 and purified by affinity chromatography on glutathione-Sepharose. Recombinant
250 MKK6 was then used to activate the p38 proteins, and subsequently removed by
251 passage through amylose resin. The enzyme activity of each p38 isoform was
252 quantified by the phosphorylation of a standard substrate, myelin basic protein. Each
253 kinase was used at an equivalent specific enzyme activity in reactions with HSF1 as a
254 substrate.

255 All p38 isoforms were able to rapidly phosphorylate HSF1 (**Fig. 6B**, black
256 bars). Quantitative analysis of incubation reactions of HSF1 with either p38 α ,
257 p38 β , p38 γ or p38 δ and Mg-[γ ³²P]-ATP revealed that p38 δ phosphorylated HSF1 at a
258 higher rate and stoichiometry than did p38 α , p38 β or p38 γ , indicating that HSF1 is a
259 better substrate for p38 δ than for any of the other p38 isoforms (**Fig. 6B**, black bars
260 and **Fig. 6C**). Parallel reactions in the absence of HSF1 showed that, under these
261 experimental conditions, there was no detectable autophosphorylation of any of the
262 kinases (not shown), and the radioactivity in these samples (as quantified by
263 scintillation counting) was identical to that of the buffer blank. Western blot analysis
264 using the S326-posphospecific antibody confirmed this conclusion and clearly
265 demonstrated that S326 was one of the phosphorylation sites (**Fig. 6D**).

266 The extent of phosphorylation of HSF1 was dependent on the kinase
267 concentration as well as the incubation time. Thus, with 6 mU/ μ l of enzyme and 60
268 min incubation (when phosphate incorporation had reached or was approaching a
269 plateau), the stoichiometry of the reaction was 2, 1.3, 0.8, and 2.2 mol/mol for the
270 α , β , γ and δ isoforms, respectively, suggesting that, under these experimental
271 conditions, p38 α , p38 β and p38 δ were able to phosphorylate at least two sites on
272 HSF1. With 0.06 mU/ μ l of enzyme and 15 min incubation, the stoichiometry of the
273 reaction for HSF1 was 0.04, 0.03, 0.03, and 0.28 mol/mol for p38 α , p38 β , p38 γ and
274 p38 δ , respectively (**Fig. 6B**, black bars), indicating that p38 δ was the most efficient
275 catalyst among the isoforms. The p38 δ -mediated phosphorylation of HSF1 was
276 inhibited by BIRB0796 (**Fig. 6E**), which inhibits all p38 MAPK isoforms (41). In
277 contrast to wild-type (WT) HSF1, under the same experimental conditions, the
278 stoichiometry of the reaction for mutant HSF1, in which S326 was replaced with
279 alanine (S326A) was 0.02, 0.02, 0.01 and 0.14 mol/mol for the p38 α , β , γ and δ
280 isoforms, respectively (**Fig. 6B**, grey bars and **Fig. 6C**), implicating S326 as one of

281 the phosphorylation sites, and confirming the existence of additional site(s) on HSF1
282 which are also phosphorylated by p38 MAPK. Interestingly, this experiment also
283 showed that, although not as robust as p38 δ , p38 γ was the most selective isoform in
284 phosphorylating S326.

285 To confirm the unusually high substrate preference of p38 δ for HSF1 relative
286 to the more widely studied p38 α/β isoforms, we carried out analogous reactions with
287 MK2 as a substrate. In comparison with p38 δ , the initial rates of activation of this
288 physiological substrate are ~20 times faster for the α or β isoforms (44). Indeed, p38 δ
289 was far less effective in phosphorylating MK2 than was p38 α (Fig. 6F). In sharp
290 contrast, p38 δ was more than 20-fold more efficient than p38 α in catalyzing the
291 phosphorylation of HSF1. As expected, the p38 α -, but not p38 δ -mediated
292 phosphorylation of all three substrates was dose-dependently inhibited by the p38 α/β
293 inhibitor SB202190.

294 As mentioned above, we employed MBP-MKK6 to obtain active bacterially
295 produced human p38 MAPK. To make absolutely certain that the phosphorylation of
296 HSF1 was not due to any residual MKK6, a new preparation of p38 δ was made in
297 parallel to a preparation of a kinase-dead mutant (D168A) version of the
298 enzyme under identical conditions. After incubation and removal of the activating
299 kinase, each protein was used at an equivalent concentration in a reaction with HSF1
300 as a substrate. Phosphorylation of HSF1 only occurred with the active, but not kinase-
301 dead p38 δ , as revealed by autoradiography and western blot analysis (Fig. 6G),
302 establishing the p38 δ enzyme as the only HSF1 kinase activity in the preparation.

303 Together, these data demonstrate that HSF1 can be phosphorylated *in vitro* by
304 all p38 MAPK isoforms at S326, and that p38 δ is the most efficient catalyst among
305 the isoforms, whereas p38 γ is the most specific. However, it is important to note that
306 S326 is not the only site of phosphorylation by these kinases as the S326A mutant
307 HSF1 was also phosphorylated, albeit with the expected reduction in stoichiometry.
308 To identify the phosphorylation sites precisely, we employed a protease-mass
309 spectrometric approach. Recombinant HSF1 was incubated with recombinant p38 α or
310 p38 δ and, after electrophoretic separation and in-gel proteolytic digestion, the
311 resulting tryptic peptides were analyzed by mass spectrometry. Under these
312 conditions, the sequence coverage was ~50%. We found that the two p38 isoforms
313 phosphorylated identical sites (Table 1), suggesting that the increased phosphate

incorporation with p38 δ □□□ due to a faster rate of phosphorylation of the same sites that were phosphorylated by p38 α and not due to phosphorylation of additional site(s). Three phosphorylated peptides were identified in each sample. The corresponding mass (m/z 2902.4659 for p38 α and m/z 2902.4683 for p38 δ) of the longest peptide was in precise agreement with the calculated molecular weight of a peptide containing phosphorylated S326 (m/z 2902.4689, R.VEEASPGRPSSVDTLLS³²⁶PTALIDSILR.E). The mass of the shorter peptides (m/z 1299.5487 and 1526.7121 for p38 α and m/z 1299.5493 and 1526.7121 for p38 δ) corresponded exactly to the molecular weight of peptides K.EEPPSPQS³⁰⁷PR.V (m/z 1299.5496) and R.VKEEPPS³⁰³PPQS³⁰⁷PR.V (m/z 1526.7130), in which both S303 and S307 were phosphorylated. Notably, S303 was found in both phosphorylated as well as unphosphorylated forms. The phosphorylation of HSF1 at S303/307 by the same kinase which phosphorylates the transcription factor at S326 was at first glance surprising, because in contrast to the activating S326 phosphorylation, phosphorylation at S303/307 is inhibitory (14-16). However, this finding provides a possible explanation for the observation that although PEITC treatment causes a concentration-dependent increase in HSF1 phosphorylation (Fig. 3 and 4A), induction of Hsp70 is lower at the higher PEITC concentration (Fig. 1B, 3A and 4A). Immunoblotting with isoform-specific antibodies showed that p38 α , p38 γ and p38 δ are well expressed in MDA-MB-231 cells, and confirmed that the levels of these kinases did not change upon exposure to PEITC (Fig. 4E).

Deletion or inhibition of p38 γ decreases the phosphorylation of HSF1 at S326 in cells. To address whether p38 γ and p38 δ play a role in the phosphorylation of HSF1 at S326 in cells, we first used the human epidermoid cancer cell line A431, in which both p38 γ and p38 δ are expressed, along with its derivatives in which p38 γ or p38 δ had been stably knocked down by more than 90%, using selective short hairpin RNA (shRNA) (Fig. 7A) (45). In comparison to the parental cells or cells deficient in p38 δ , the heat shock-mediated phosphorylation of HSF1 at S326 was reduced by 60% in cells lacking p38 γ (Fig. 7B), in close agreement with the high selectivity of this isoform for the S326 phosphorylation *in vitro* (Fig. 6B-D). Treatment with the p38 α / β -specific inhibitor SB202190 had no further effect, indicating that p38 α and p38 β do not contribute significantly to the phosphorylation of S326 in these cells (Fig. 7B).

348 Similar results were obtained in MDA-MB-231 cells: shRNA-mediated
349 knockdown (by more than 90%) of p38 γ led to a substantial reduction (by ~50%) in
350 the phosphorylation of HSF1 at S326 at basal cell culture conditions, whereas the
351 knockdown of p38 δ did not have this effect (Fig. 7C). The knockdown of p38 γ led a
352 corresponding decrease in the levels of Hsp70 (Fig. 7C). Interestingly, the levels of
353 Hsp70 were also reduced upon p38 δ knockdown, even though the phosphorylation of
354 HSF1 at S326 was not affected. These data suggest that p38 δ might be involved in
355 catalyzing the phosphorylation of other (than S326) sites, which activate HSF1,
356 consistent with the highest stoichiometry of the reaction of this p38 isoform *in vitro*
357 with both WT and S326A mutant HSF1 (Fig. 6B,C); the identity of these potential
358 sites is presently unknown.

359 The conclusion that p38 α and p38 β do not contribute significantly to the
360 phosphorylation of S326 in A431 cells was further supported by studies in PEITC-
361 treated MDA-MB-231 cells, where SB202190 either had no effect (at the high
362 concentration of PEITC) or even enhanced by 2.5-fold (at the low concentration of
363 PEITC) the levels of pS326 HSF1 after exposure to the isothiocyanate (Fig. 6D). The
364 activation of p38 α/β by PEITC and the efficacy of SB202190 were confirmed by
365 monitoring the levels of phosphorylated (at T334) MK2 (Fig. 7D). Finally, we used
366 BIRB0796, which inhibits all four p38 MAPK isoforms (41). Pre-treatment with
367 BIRB0796 for 1 h reduced the phosphorylation of S326 in PEITC-treated MDA-MB-
368 231 cells (Fig. 7E). Together, these findings strongly suggest that p38 γ is the
369 principal p38 MAPK isoform responsible for the phosphorylation of HSF1 at S326 in
370 cells.

371 Phosphorylation at S326, but not at any of the other serine residues identified
372 by Guettouche *et al.*, has been shown to affect the heat shock-induced transcriptional
373 activity of HSF1 without affecting the trimerization of nuclear translocation of the
374 transcription factor. In agreement, we found that both purified recombinant wild-type
375 and S326A mutant HSF1 have the propensity to form dimers and trimers
376 spontaneously (Fig. 6A). By use of quantitative high content imaging, we examined
377 the nuclear and cytoplasmic distribution of wild-type, S326A or S326E mutant GFP-
378 HSF1 fusion proteins after their ectopic expression in HSF1-knockout MEFs, and did
379 not observe any significant differences among the genotypes (Fig. 8). Notably
380 however, these results should be interpreted with caution: it is well documented that

in *C. elegans*, ectopic expression of HSF1 produces a gain-of-function phenotype (46, 47), indicating that any level of overexpression of HSF1 may not accurately reflect the physiological properties of the endogenous protein.

Discussion

By use of mass spectrometry and protein sequencing, Guettouche *et al.* (19) found that in cells subjected to heat shock, human HSF1 is phosphorylated at 12 serine residues: S121, S230, S292, S303, S307, S314, S319, S326, S344, S363, S419, and S444. More recently, using mass spectrometry-based proteomics, Xu *et al.* (48) reported the phosphorylation at five additional serine residues (S127, S195, S216, S320, and S368), and at four threonine residues (T142, T323, T367, and T369). The functional significance of most threonine phosphorylations is unknown, except for T142, the phosphorylation of which by casein kinase 2 (CK2) has been reported to increase the transcriptional activity of HSF1 (49). It is well established that phosphorylations at S303/307, S121 and S363 inhibit the function of the transcription factor and are involved in the attenuation phase of the heat shock response (14-16, 18), whereas phosphorylation at S216 by Polo-like kinase 1 (PLK1) promotes the ubiquitination and degradation of HSF1 during mitosis (50). Curiously, PLK1 also phosphorylates HSF1 at S419, but in contrast to the inhibitory S216 phosphorylation, phosphorylation at S419 is activating and promotes the nuclear translocation of the transcription factor (51). Phosphorylation at S320 by protein kinase A (PKA) also activates HSF1 (52). Another activating phosphorylation occurs at S230; it is catalysed by calcium/calmodulin-dependent protein kinase II (CaMKII) and enhances the magnitude of the response upon heat stress (53). Although the DNA-binding ability of the S230A mutant of HSF is retained, its transcriptional activity is reduced by ~50% in comparison with wild-type HSF1.

Phosphorylation at S326 is a hallmark for HSF1 activation, and several studies have attempted to identify the kinase(s) phosphorylating this site. It was reported that the mechanistic target of rapamycin (mTOR) is able to catalyze the phosphorylation of HSF1 at S326 (20). However, PEITC inhibits mTOR (54). In full agreement, we also found that the mTOR pathway was inhibited by PEITC, as evidenced by the decreased phosphorylation of the ribosomal subunit S6 at S235/236 (Fig. 4B). Overall, our data presented in this contribution imply that mTOR is not the primary kinase

414 responsible for the phosphorylation of S326 on HSF1 in response to treatment with
415 PEITC, and further implicate the family of proline-directed p38 MAPK as highly
416 efficient catalysts, which phosphorylate HSF1 rapidly and stoichiometrically. Notably
417 however, neither pharmacological inhibition of p38 MAPK by small molecule
418 inhibitors or genetic downregulation of p38 γ or p38 δ eliminated completely the
419 phosphorylation of HSF1 at S326 (Fig. 7). These results imply that although p38 γ is
420 the principal isoform within the p38 MAPK family that phosphorylates this site,
421 inactivation of p38 γ allows for compensation by other kinases. One such candidate is
422 JNK1/2, which is also activated by PEITC, albeit at a later time point in comparison
423 with p38 MAPK (Fig. 4D). It has been reported that p38 MAPK engage in feedback
424 control loops that suppress the activities of upstream MAP kinase kinase kinases
425 (MAP3Ks), which participate in the activation JNK, and by disrupting these feedback
426 control loops, inhibition of p38 leads to hyperactivation of JNK (55). The possibility
427 that JNK1/2 could phosphorylate HSF1 at S326 requires a further study.

428 Interestingly and unexpectedly, we found that in addition to S326, p38 MAPK
429 can also catalyze the phosphorylation of HSF1 at S303/307. In contrast to the
430 activating function of the S326 phosphorylation, phosphorylation at S303/307 inhibits
431 the function of HSF1 (14-16). Although surprising, the fact that the same kinase can
432 catalyze phosphorylation of two distinct sites on HSF1 with opposing functional
433 consequences is not unprecedented. As mentioned above, phosphorylation at S216 by
434 PLK1 inhibits HSF1, whereas phosphorylation at S419 by the same kinase activates
435 the transcription factor (50, 51). Our results imply that either p38 MAPK
436 phosphorylate S326 at a faster rate than at S303/307, thus giving a “window” of HSF1
437 activation due to S326 phosphorylation before the repressive effect of S303/307
438 phosphorylation takes place, or alternatively, that there is a threshold of MAPK p38
439 activation below which S326 is the principal target, and above which the S303/307
440 phosphorylation becomes dominant. The second possibility is supported by the fact
441 that induction of Hsp70 is lower upon treatment with the higher (20 μ M) compared to
442 the lower (10 μ M) concentration of PEITC, whereas the extent of HSF1
443 phosphorylation is dependent on the dose of PEITC. In addition, the identity of the
444 phosphatases involved may also influence the relative turnover rates of
445 phosphorylation at each site. Dissecting these possibilities requires better tools for

quantitation of relative stoichiometry of phosphorylation at S303/307 vs. S326 in different cell compartments.

Previous investigations have suggested the possible involvement of MAPK signaling in the activation of HSF1. Thus, loss of the tumor suppressor neurofibromatosis type 1 (NF1) leads to activation of MAPK signaling and HSF1 (6). Chronic exposure of rodent fibroblast cells to heat stress causes phosphorylation of p38 MAPK and induction of Hsp70 (56, 57). In addition, the anti-inflammatory agent sodium salicylate, has been reported to activate p38 MAPK, promote HSF1 DNA binding and transcriptional activity, and induce Hsp70 expression (58). Most recently, it was reported that HSF1 physically interacts and is phosphorylated by MEK (42). However, to our knowledge, there are no prior publications linking HSF1 phosphorylation at S326 directly with p38 MAPK activation. In our study, the identification of p38 MAPK as one family of kinases, which phosphorylate HSF1 at S326 was greatly facilitated by the observation that PEITC is an exceptionally robust activator of HSE-dependent transcription (**Fig. 1F**). PEITC shares the ability to induce the heat shock response with celastrol (59, 60), another natural product which like PEITC, has a characteristic chemical signature, reactivity with sulfhydryl groups (61). Notably, in a screen comprising ~900 000 small molecules, Calamini *et al.* (34) discovered new classes of HSF1 activators, all of which, although structurally diverse, bear the same chemical signature. We propose that this chemical property underlies the ability of PEITC to both inhibit Hsp90 as well as activate p38 MAPK. **Finally, pharmacological targeting of p38 γ and p38 δ has been recently proposed for the treatment of autoimmune and inflammatory diseases, as well as cancer (62). As HSF1 activation supports malignant transformation (5), this approach holds promise for targeting HSF1 for cancer treatment.**

Materials and Methods

Materials. All general chemicals and reagents were of analytical grade and obtained from Sigma-Aldrich (Dorset, United Kingdom). PEITC was prepared as a stock solution in acetonitrile and diluted 1:1,000 in the cell culture medium before treatment. The concentration of the solvent was maintained at 0.1% (v/v) in all wells. The p38 α/β MAPK inhibitor SB202190 was purchased from SYNkinase. The JNK inhibitor JNK-In-8 was a kind gift from Dario Alessi (University of Dundee).

479

480 **Cell culture.** MDA-MB-231 cells were from ATCC. HeLa-HSE-luc cells (34) were
481 a generous gift from Richard I. Morimoto (Northwestern University, USA). Mouse
482 embryonic fibroblasts (MEF) from wild-type or HSF1-knockout mice were isolated as
483 described previously (63). The human epidermoid cancer cell line A431 and the
484 production and transduction of lentivirus short hairpin RNA to generate stable clones,
485 which do not express p38 γ or p38 δ , have been described (45). All cell lines were
486 maintained at 5% CO₂ in air at 37°C and were cultured in Dulbecco's Modified Eagle
487 Medium (DMEM) supplemented with 10% (v/v) heat-inactivated FBS. The medium
488 in which HeLa-HSE-luc cells were grown also contained 100 μ g/mL G418
489 (Invitrogen), whereas the medium for MEF cells was additionally supplemented with
490 non-essential amino acids and 50 U/ml penicillin/streptomycin.

491

492 **Western blotting.** Cells grown in 6-well plates were washed twice with phosphate
493 buffered saline (PBS) and lysed in 150 μ l of either RIPA buffer [50 mM Tris-Cl pH
494 7.5, 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 1% NP-40 (v/v), 0.1% SDS
495 (w/v) and 1 mM EDTA], containing 1 protease inhibitor cocktail tablet (Roche) per
496 10 ml of buffer] or SDS lysis buffer [50 mM Tris-Cl pH 6.8, 2% (w/v) SDS, 10%
497 (v/v) glycerol and 0.005% bromophenol blue). The lysates derived from RIPA buffer
498 were transferred into 1.5-ml Eppendorf tubes which were placed on a rotator at 4°C
499 for 30 min. The cell debris was then removed by centrifugation at 16,300 x g for 10
500 min at 4°C, and the supernatant was transferred to a new tube. The lysates derived
501 from the SDS lysis buffer were subjected to sonication at 20% amplitude for 20 sec.
502 The BCA assay (Thermo) was used to determine protein concentrations. Proteins
503 were resolved by SDS/PAGE, transferred to immobilon-P membranes, and probed
504 with specific antibodies against Hsp70 (mouse monoclonal, 1:1,000, StressMarq,
505 York, United Kingdom), Hsp90 (mouse monoclonal, 1: 5,000, BD Biosciences, New
506 Jersey, USA), HER2 (rabbit polyclonal, 1:500, Millipore, California, USA), RAF1
507 (rabbit polyclonal, 1:200, Santa Cruz, California, USA), HSF1 (rabbit polyclonal,
508 1:1,000, Enzo Life Sciences, Exeter, United Kingdom), pS326-HSF1 (rabbit
509 polyclonal, 1:10,000, Abcam, Cambridge, United Kingdom), p38 MAPK (rabbit
510 polyclonal, 1:1,000, Cell Signaling, Massachusetts, USA), pp38 MAPK (rabbit
511 polyclonal, 1:1,000, Cell Signaling, Massachusetts, USA), JNK1/2 (rabbit polyclonal,
512 1:1,000, Cell Signaling, Massachusetts, USA), pJNK1/2 (rabbit polyclonal, 1:1,000,

513 Biosource Europe, Nivelles, Belgium), pERK1/2 (rabbit polyclonal, 1:1,000, Cell
514 Signaling, Massachusetts, USA), pT334-MK2 (rabbit polyclonal, 1:1,000, Cell
515 Signaling, Massachusetts, USA), and pS235/6 S6 (rabbit polyclonal, 1:5,000, Cell
516 Signaling, Massachusetts, USA). Isoform-specific p38 γ and p38 δ MAPK antibodies
517 were from the Division of Signal Transduction Therapy (DSTT), and were used at a
518 concentration of 1 μ g/ml. Equal loading was confirmed by probing the blots with
519 antibodies against GAPDH (rabbit polyclonal, 1:5,000) or β -actin (mouse monoclonal,
520 1:10,000), both from Sigma, Dorset, United Kingdom, or Lamin A (rabbit polyclonal,
521 1:1,000, GeneTex, Irvine, CA, USA). The western blots shown are representative of
522 at least three independent experiments.

523

524 **Nuclear-cytoplasmic separation.** MDA-MB-231 cells (10^6 per dish) were plated in
525 6-cm dishes and treated for the indicated periods of time with 0.1% (v/v) acetonitrile
526 or PEITC. The REAP method described by Suzuki *et al.* (64) was used to obtain
527 separate cytoplasmic and nuclear fractions. In short, cells were washed twice with ice-
528 cold phosphate buffered saline (PBS, pH 7.5), collected in 500 μ l of ice-cold PBS,
529 transferred to Eppendorf tubes, and subjected to centrifugation at 10,000 x g for 30
530 sec at room temperature. Next, the supernatant was discarded and the pellet was
531 resuspended in 450 μ l of ice-cold 0.1% NP-40 (v/v) in PBS. The lysates were then
532 subjected to a further centrifugation at 10,000 x g for 30 sec at room temperature. The
533 supernatant was collected as the cytoplasmic fraction. One volume of 5X sample SDS
534 loading buffer [250 mM Tris-Cl pH 6.8, 10% (v/v) sodium dodecyl sulphate, 50%
535 (v/v) glycerol and 0.025% (w/v) bromophenol blue] was added to four volumes of the
536 cytoplasmic fraction and the samples were heated for 5 min at 100°C and subjected to
537 SDS/PAGE. The remaining pellet containing the nuclear fraction was washed twice
538 with ice cold 0.1% NP-40 (v/v) in PBS and dissolved in 1X sample loading buffer [50
539 mM Tris-Cl pH 6.8, 2% (v/v) sodium dodecyl sulphate, 10% (v/v) glycerol and
540 0.005% (w/v) bromophenol blue] and heated for 5 min at 100°C. The nuclear
541 fractions were sonicated before subjecting them to SDS-PAGE.

542

543 **Quantitative real-time PCR.** The primers and probes for quantifying the levels of
544 the mRNA species were from Applied Biosystems (*hspa1a*: Mm01159846_s1; *HER2*:
545 HS01001580_m1 and *RAFI*: HS00234119_m1). Cells (2×10^5 per well) were seeded

546 in 6-well plates. After 24 h, the cells were exposed to vehicle (0.1% acetonitrile) or
547 PEITC for a further 8 (MEFs) or 16 h (MEFs and MDA-MB-231 cells). After cell
548 lysis, total RNA was extracted using RNeasy Kit (Qiagen Ltd.), and 500 ng total RNA
549 was reverse transcribed into cDNA with Omniscript Reverse Transcription Kit
550 (Qiagen Ltd.). Real-time PCR was performed on Applied Biosystems 7900HT Fast
551 Real-Time PCR System. The data were normalized using β -actin (mouse ACTB,
552 Applied Biosystems, Mm00607939_s1) as an internal control.

553
554 **Luciferase assay.** HeLa-HSE-luc cells (10^5 per well) were seeded in each well of a
555 24-well plate, and 24 h later treated with PEITC or 0.1% (v/v) acetonitrile vehicle for
556 8-, 16-, 24-, or 48 h. Cells were washed twice with 0.1% PBS, and 100 μ l of 1X
557 reporter lysis buffer (Promega) was added to each well. The plate was placed at -20°C
558 for a minimum for 2 h, and then transferred to thaw on a shaker at room temperature
559 for 30 min. Cell lysates were collected into Eppendorf tubes and subjected to
560 centrifugation at $15,000 \times g$ for 2 min at 4°C . Luciferase activity was measured in 10
561 μ l of cell lysate in opaque 96-well plates (Corning) using a microplate-reader based
562 luminometer (Orion II, Berthold), and normalized for protein concentration
563 determined by the Bradford's assay (BioRad).

564
565 **ATP-binding assay.** MDA-MB-231 cells (0.5×10^6 per dish) were seeded in 6-cm
566 dishes. After 24 h, the cells were treated for a further 24 h with 0.1% acetonitrile as
567 the vehicle control for sulfoxythiocarbamate alkyne (STCA, 75 μM) and PEITC (20
568 μM) treatments, or with 0.1% DMSO as the vehicle control for the geldanamycin
569 (GA, 1 μM) and celastrol (CL, 0.8 μM) treatments. Cells were harvested by scraping
570 into 300 μ l of lysis buffer [10 mM Tris pH 7.5, 150 mM NaCl, 0.25% NP40, with one
571 protease inhibitor tablet (Roche) per 10.0 ml of buffer], frozen, thawed, and lysed for
572 30 min at 4°C . ATP-agarose beads (Jena Bioscience) were washed with the
573 incubation buffer (10 mM Tris pH 7.5, 150 mM NaCl, 20 mM MgCl_2 , 0.05% NP40, 1
574 mM DTT). Cell lysates (200 μg total protein) were added to a suspension of 30 μ l of
575 beads in 1.25 ml of buffer, and the samples were incubated rotating overnight at 4°C .
576 The beads were collected by centrifugation and washed three times with the
577 incubation buffer. SDS loading buffer (10 μ l) and incubation buffer (40 μ l) were
578 added to the beads, and the samples were incubated for 5 min at 100°C . The beads

579 were pelleted by centrifugation, and the supernatants were collected and subjected to
580 western blot analysis.

581

582 **Detection of HSF1 trimerization.** MDA-MB-231 (2×10^6 per dish) cells were grown
583 on 10-cm dishes for 24 h and treated with 0.1% acetonitrile or 20 μ M PEITC for a
584 further 3 h. Cells were then washed twice with PBS. 10 ml of 0.4% paraformaldehyde
585 (w/v) in PBS (0.4% PFA-PBS) was added to the dishes over 10 min, where fresh
586 0.4% PFA-PBS was added every 5 min. Next, the PFA-PBS was removed and the
587 reaction was quenched with the addition of 3 ml of ice-cold 1.25 M Glycine-PBS.
588 After washing twice with PBS, nuclear and cytoplasmic fractions were obtained. Cells
589 were lysed in buffer A [10 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-Cl pH 7.5, 0.5% (v/v)
590 NP-40, 1 mM DTT, one EDTA-free complete mini protease inhibitor cocktail tablet
591 (Roche) and one phos-STOP tablet (Roche) per 10 ml of buffer]. The lysates were
592 subjected to centrifugation at $1,000 \times g$ for 5 min at 4°C. The supernatant containing
593 the cytoplasmic fraction was transferred to a fresh Eppendorf tube where one volume
594 of 5X SDS sample loading buffer was added to four volumes of the cytoplasmic
595 fraction. The pellet containing the nuclear fraction was washed three times with the
596 buffer A before dissolving it in 1X sample loading buffer [50 mM Tris-Cl pH 7.4, 2%
597 (w/v) SDS, 10% (v/v) glycerol, and 0.005% (w/v) bromophenol blue]. The nuclear
598 fractions were subjected to sonication. Both the cytoplasmic and the nuclear fractions
599 were subjected to SDS-PAGE before immunoblotting.

600

601 **Co-immunoprecipitation.** MDA-MB-231 (4×10^6 per dish) cells were grown on 10-
602 cm dishes for 24 h, and the treated with 0.1% DMSO or 20 μ M PEITC for 45 min.
603 The dishes with cells were placed on ice and washed twice with ice-cold PBS. Protein
604 G Dynabeads (30 μ l slurry, from Invitrogen) were washed twice for 5 min with PBS
605 and incubated with 1 μ g of mouse monoclonal HSF1 antibody (Santa Cruz) for 1 h at
606 room temperature, after which the beads were washed three times every 10 min with
607 PBS. Cells were lysed with 1.0 ml ice-cold CO-IP buffer (150 mM NaCl, 50 mM
608 Tris-Cl, pH 7.4, 1 mM EDTA, 1% NP-40, 0.1% w/v sodium deoxycholate)
609 supplemented with one EDTA-free protease cocktail inhibitor tablet (Roche) and one
610 phosphatase inhibitor tablet (PhoSTOP Roche). Cell lysates were passed through a
611 23-gauge needle 10 times before they were clarified by centrifugation at 4°C for 30

min at 16,000 x g. Fifty μ l of the clarified lysate (IP sample) was transferred to a fresh Eppendorf tube to serve as an input sample. To pre-clear the IP sample, 30 μ l of Protein G Dynabeads slurry was washed twice for 5 min with PBS, and the beads were added to each of the IP sample (containing 0.8-1.0 mg protein), and incubated for 1 h at 4°C on a tube rotator. Subsequently, the Protein G Dynabead-antibody conjugate was added to the pre-cleared IP sample and incubated for 16 h at 4°C on a tube rotator. The immunoprecipitated complexes were washed three times with ice-cold CO-IP buffer every 10 min, and were eluted from the beads by adding 70 μ l of 1X LDS buffer (Invitrogen) and heating the sample at 70°C for 10 min. After cooling, 7 μ l of sample reducing agent (SRA, Invitrogen) was added to the sample, and incubated for 15 min at room temperature. Immunoprecipitated proteins (35 μ l) were resolved by electrophoresis. Antibodies against Hsp90 (monoclonal, BD Biosciences) and HSF1 (rabbit polyclonal, Enzo Life Sciences) were used for detection of the respective proteins.

Generation of p38 γ and p38 δ stable knockdown cell lines. p38 γ and p38 δ expression was reduced by RNA interference using Mission shRNA constructs (Sigma; plasmid clone IDs TRCN0000006145 and TRCN0000006147 for p38 γ , and TRCN0000000827 and TRCN0000009979 for p38 δ). A lentivirus containing the control pLKO.1 or the shRNA plasmids was used to infect MDA-MB-231 cells. To produce the virus, HEK293T cells were transfected using Lipofectamine 2000 (Invitrogen) with empty pLKO.1-puro vector or the shRNA constructs against p38 γ or p38 δ , together with the packaging vectors (psPAX2 and pMD2.G) in serum-reduced medium. On the following day, the medium was replaced with complete DMEM, and after 24 h, the lentivirus-containing supernatant was collected, filtered, and used to transduce MDA-MB-231 cells. Cells containing the shRNA plasmid were selected, expanded and maintained with supplementation of puromycin (2 mg/ml) for approximately three weeks, during which time cell lysates were collected every three to four days to ensure the respective p38 expression levels were reduced throughout the selection period.

Expression and purification of recombinant hexahistidine-tagged HSF1. Full-length HSF1 cDNA was amplified by PCR from a plasmid obtained from Addgene

(Plasmid ID: #32537, in which the cDNA sequence was found to have a nucleotide substitution at position 1343 from a C to T, leading to a change from P to L at position 448 in the protein sequence) and subcloned into the bacterial expression vector pet15b using NdeI and XhoI. Following transformation into *E. coli* (BL21(DE3)pLysS), the cells were grown to an optical density at 600 nm of 0.6, and induced for a further 3.5 h at 37°C with 400 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The induced cells were harvested by centrifugation and resuspended in extraction buffer [20 mM Tris-Cl pH 7.9, 150 mM NaCl, 5 mM imidazole and 0.01% (v/v) IGEPAL CA-630]. After freezing and thawing, the cells were disrupted by sonication for 5 min on ice. Cell debris were then cleared by centrifugation at 10,000 x g for 15 min at 4°C. The resultant supernatant was left on ice for 30 min before applying to nickel agarose resin (His-TrapTMHP, GE Healthcare). The resin was washed with 20 mM Tris-Cl pH 7.9, 150 mM NaCl, 5 mM imidazole. The supernatant (20 ml) was then incubated for 1 h at 4°C with 1 ml resin. After three washes with buffer, the protein was eluted with 2 ml of 20 mM Tris-Cl pH 7.9, 150 mM NaCl, 250 mM imidazole. To remove the imidazole, the preparation was dialyzed in 50 mM Tris-Cl pH 7.4, 150 mM NaCl. Mutant S326A HSF1 was generated by site-directed mutagenesis of the plasmid vector pet15b containing the HSF1 cDNA by using the primers 5'-GTGGACACCTCTTGGCCCCGACCGCCCTCATTG-3' and 5'-CAATGAGGGCGGTCGGGGCCAAGAGGGTGTCCAC-3' and the QuikChange® II Mutagenesis kit (Stratagene). The hexahistidine-tagged mutant S326A HSF1 recombinant protein was generated using the method described above.

High content microscopy and analysis. HSF1-knockout MEFs were seeded in black-walled 96-well plates (Corning Costar 3904) at 4 x 10³ cells/well and transfected with 50ng/well GFP-tagged wild-type or S326A or S326E mutants of HSF1 using Lipofectamine LTX reagent (Invitrogen), using 8 replicate wells per condition. Twenty-four hours after transfection, cells were fixed using 4% paraformaldehyde in PBS for 10 min at room temperature, and permeabilised using methanol at -20°C for 5 min. Cells were blocked using 2.5% normal goat serum in PBS/0.1% sodium azide, and counterstained using rabbit anti-ERK1/2 mAb (clone 137F5, Cell Signaling Technology) and Alexa 546 labelled highly cross adsorbed

678 goat anti-rabbit secondary antibody (Invitrogen). DNA was labelled with 300 nM
679 DAPI (Sigma) in PBS and images were acquired using an IN Cell Analyzer 2000
680 robotic fluorescence microscope (GE Healthcare) using a 20x lens to capture 4 fields
681 per well for each fluorophore (DAPI, GFP and Alexa 546) using 2x2 pixel binning to
682 maximise signal/noise. Images were analyzed using a custom algorithm constructed
683 within IN Cell Developer software (GE Healthcare), using DAPI and ERK1/2 images
684 to identify nuclear and cytoplasmic regions, respectively, in order to assess
685 fluorescence distribution within the GFP channel.

686

687 **Kinase assays.** The incubation mixtures contained purified recombinant kinase (at a
688 specific activity of either 6 mU/ μ l or 0.06 mU/ μ l), recombinant HSF1 (1 μ g) substrate,
689 10 mM MgCl₂, 0.1 mM [γ -³²P]-ATP (approximately 0.5 x 10⁶ cpm/nmol), and kinase
690 buffer [50 mM Tris-Cl, 0.03% (v/v) Brij-35, 0.1% (v/v) β -mercaptoethanol] in a total
691 volume of 50 μ l. The kinase assays were performed at 30°C. At the times indicated, a
692 15- μ l aliquot of each incubation mixture was removed, the reaction was terminated by
693 the addition of SDS gel loading buffer, the sample was loaded on SDS-PAGE, and the
694 excess [γ -³²P]-ATP was removed by electrophoresis. The gels were dried and
695 subjected to autoradiography. Protein-containing gel pieces (visualized by staining
696 with Coomassie Brilliant Blue) were then excised, and phosphate incorporation into
697 HSF1 was quantified by scintillation counting.

698 Cold assays were performed in an analogous manner using purified
699 recombinant kinase (at a specific activity of 0.06 mU/ μ l), recombinant HSF1 (1 μ g)
700 substrate, MgCl₂ (10 mM), and ATP (0.1 mM) instead of [γ -³²P]-ATP. For
701 identification of the phosphorylated sites, the gel bands were excised, reduced with
702 DTT (10 mM), alkylated with iodoacetamide (50 mM), and digested overnight (16 h)
703 with trypsin (Modified Sequencing Grade, Roche) at 30°C. The resulting peptides
704 were extracted from the gel, dried in a SpeedVac concentrator (Thermo Scientific),
705 resuspended in 10 μ l 5% formic acid, and diluted five times. Any residual particles
706 were removed by centrifugation, the samples were then transferred to HPLC vials,
707 and analyzed by LC/MS/MS on Ultimate3000 RSLCnano System (Thermo Scientific)
708 coupled to a LTQ OrbiTrap VelosPro (Thermo Scientific) with EasySpray source.
709 The data files were analyzed with Proteome Discoverer (Ver. 1.4.1) using Mascot

710 (Ver. 2.4.1) as the search engine using Protein specific database (HisTag-HSF1) and
711 IPI-Human (ipi.HUMAN.v3.87) database.

712

713 **Statistical analysis.** Values are means \pm 1 SD. The differences between groups were
714 determined by Student's t-test. Analyses were performed using Excel (Microsoft
715 Corp.).

716

717 **Acknowledgments**

718 We thank Calum Saunders for participating in the early stages of this project, Richard
719 I. Morimoto (Northwestern University, USA) for the generous gift of HeLa-luc cells,
720 Stuart Calderwood (Harvard Medical School, USA) for mammalian expression
721 plasmids encoding HSF1, Dario Alessi (University of Dundee) for the JNK inhibitor
722 JNK-IN-8, and Young-Hoon Ahn (Wayne State University, USA) for
723 sulfoxythiocarbamate alkyne (STCA). We are extremely grateful to the Medical
724 Research Institute of the University of Dundee, the BBSRC (BB/J007498/1) and
725 Ministerio de Economia y Competitividad (MINECO) (SAF2013-45331-R) for
726 financial support.

727 **Figure Legends**

728 **Figure 1. PEITC is a robust inducer of the heat shock response.** (A) Chemical
729 structures of allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), and
730 phenethyl isothiocyanate (PEITC). (B, C) MDA-MB-231 cells (2.5×10^5 per well)
731 (B) or mouse embryonic fibroblasts (MEF, 2×10^5 per well) (C) growing in 6-well
732 plates were exposed to vehicle (0.1% acetonitrile), AITC, BITC, or PEITC for 16 h.
733 Cells were lysed in RIPA buffer, proteins were resolved by SDS/PAGE, transferred to
734 immobilon-P membranes, and probed with an antibody against Hsp70. The levels of
735 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as loading control. (D)
736 Wild-type MEF cells (2×10^5 per well) in 6-well plates were exposed to vehicle
737 (0.1% acetonitrile) or PEITC for 8 or 16 h. Cells were lysed, total RNA was extracted,
738 and reverse transcribed into cDNA. The mRNA levels for *hspa1a* were quantified
739 using real-time PCR. The data were normalized using β -actin as an internal control.
740 (E) MDA-MB-231 cells (2.5×10^5 per well) growing in 6-well plates were exposed to
741 vehicle (0.1% acetonitrile) or 20 μ M PEITC for 3 h. Cells were then fixed with 0.8%
742 (v/v) paraformaldehyde. Cell lysates were subjected to nuclear (N) and cytoplasmic
743 (C) separation, proteins were resolved by SDS/PAGE (10% gel), transferred to
744 immobilon-P membranes, and probed with an antibody against HSF1. The levels of
745 lamin B2 and GAPDH served as fraction purity indicators and as loading controls. (F)
746 HeLa-HSE-luc cells (1.3×10^5 per well) stably transfected with the luciferase gene
747 under the control of the *HSP70.1* promoter, were grown in 12-well plates and exposed
748 to vehicle (0.1% acetonitrile) or PEITC. Luciferase activity was determined in cell
749 lysates. The relative luminescence units (RLU) were quantified and normalized with
750 respect to the vehicle control treatment. Data represent means \pm SD and are expressed
751 as ratio of the relative transcripts in treated over control samples.

752

753 **Figure 2. PEITC inhibits Hsp90.** (A) MDA-MB-231 cells (2.5×10^5 per well) in 6-
754 well plates were treated with vehicle (0.1% acetonitrile) or PEITC for 24 h. The levels
755 of HER2 and RAF1 were detected by western blot analysis. The levels of β -actin
756 served as loading control. (B) MDA-MB-231 cells (0.5×10^6 per dish) were grown in
757 6-cm dishes. After 24 h, the cells were treated for a further 24 h with 0.1% acetonitrile
758 (ACN) as the vehicle control for sulfoxythiocarbamate alkyne (STCA) and PEITC
759 treatments, or with 0.1% DMSO as the vehicle control for the geldanamycin (GA) and

celastrol (CL) treatments. Cells were lysed and subjected to ATP pulldown using ATP-agarose beads. For the ATP pull-down and input samples, Hsp90 or GAPDH were detected by western blot analyses. (C) MDA-MB-231 cells (2.5×10^5 per well) were grown in 6-well plates and treated with vehicle (0.1% acetonitrile) or PEITC for 45 min. cells were lysed and subjected to immunoprecipitation with an anti-HSF1 antibody, and then immunoblotted with an anti-Hsp90 antibody. An aliquot of total lysate was subjected to immunoblot analysis with anti-Hsp90 and anti-HSF1 antibodies.

Figure 3. PEITC causes phosphorylation of HSF1 at S326. MDA-MB-231 cells (2.5×10^5 per well) in 6-well plates were treated with vehicle (0.1% acetonitrile) or PEITC for either 24 h (A) or for the indicated periods of time (B). In (A), the levels of pS326 HSF1, total HSF1, and Hsp70 were detected by western blot analysis in cell lysates, and the levels of β -actin served as loading control. In (B), the levels of pS326 HSF1 were detected by western blot analysis in cytoplasm and nucleus following nuclear-cytoplasmic separation. The levels of lamin A and GAPDH served as fraction purity indicators and as loading controls.

Figure 4. PEITC activates p38 and JNK1/2 MAPK, and inhibits mTOR. MDA-MB-231 cells (2.5×10^5 per well) growing in 6-well plates were treated with vehicle (0.1% acetonitrile) or PEITC for either 24 h (A, B), 3 h (C, E) or for the indicated periods of time (D). The levels of HSF1, pS326 HSF1, pS235/6 S6, Hsp70, the p38 isoforms α , γ , and δ , phosphorylated p38 (pp38), phosphorylated p38 α (pp38 α), JNK1/2, and phosphorylated JNK1/2 (JNK1/2), were detected by western blot analysis.

Figure 5. The MEK inhibitor U0126 reduces the levels of heat-shock induced phosphorylation of HSF1 at S326 after a 24-h pre-treatment, but 1-h pre-treatment has no effect. MDA-MB-231 cells (2.5×10^5 per well) in 6-well plates were pre-treated with U0126 for either 24 h (A) or 1 h (B), and subsequently subjected to heat shock (HS). The levels of HSF1 and pS326 HSF1 were detected by western blot analysis. The levels of β -actin served as loading control.

793 **Figure 6. p38 MAPK phosphorylate HSF1 *in vitro*.** (A) Electrophoretic mobility
794 (NuPAGE NoVex Bis-Tris 4-12% gradient gel) of recombinant hexahistidine-tagged
795 HSF1 wild-type (WT) and S326A mutant. (B-G) Purified activated recombinant p38
796 MAPK isoforms (0.06 mU/ μ l) were incubated with recombinant wild-type (WT),
797 S326A mutant HSF1, MK2, or myelin basic protein (MBP) (all at 0.1 μ g/ μ l) at 30°C
798 for 15 min in the presence of 10 mM MgCl₂ and 0.1 mM [γ -³²P]-ATP. Identical
799 reactions were carried out in the presence of increasing concentrations of the p38 α / β
800 inhibitor SB202190 or BIRB0796, which inhibits all p38 isoforms. The reactions
801 were terminated by addition of SDS gel loading buffer, the samples were loaded on
802 SDS/PAGE, and the excess [γ -³²P]-ATP was removed by electrophoresis. The gels
803 were dried and subjected to autoradiography (C, E-G). After staining with Coomassie
804 Brilliant Blue, the protein bands were excised and the incorporated radioactivity (B)
805 was determined by scintillation counting. *, $p < 0.05$. (D, F) Purified activated
806 recombinant p38 α (0.06 mU/ μ l) was incubated with recombinant wild-type (WT) or
807 S326A mutant HSF1 (0.1 μ g/ μ l) at 30°C for 15 min in the presence of 10 mM MgCl₂
808 and 0.1 mM ATP. The reactions were terminated by addition of SDS gel loading
809 buffer, the samples were loaded on SDS/PAGE, and the phosphorylation of HSF1 at
810 S326 and the levels of total HSF1 were detected by western blotting.

811

812 **Figure 7. Deletion or inhibition of p38 γ MAPK reduces the levels of pS326 HSF1**
813 **in cells.** (A) Immunoblotting for p38 γ and δ in A431 cells, which either express both
814 p38 γ and p38 δ (WT), or in which p38 γ or p38 δ had been stably knocked down using
815 selective shRNA. (B) A431 cells (5 x 10⁵ per well, WT or p38 γ - or p38 δ -deficient)
816 were pre-incubated for 1 h with vehicle (0.1% acetonitrile) or SB202190, and exposed
817 to heat shock (42 °C) for a further 1 h. (C) p38 γ - or p38 δ was stably knocked down in
818 MDA-MB-231 cells using selective shRNA. The levels of total HSF1, HSF1
819 phosphorylated at S326, total p38, p38 γ , and p38 δ , and Hsp70 were detected by
820 western blot analysis. (D, E) MDA-MB-231 cells (5 x 10⁵ per well) grown in 6-well
821 plates were pre-treated with vehicle (0.1% acetonitrile), SB202190 or BIRB0796 for 1
822 h, and subsequently either treated with vehicle (0.1% acetonitrile) or PEITC for a
823 further 1.5 h. HSF1 phosphorylated at S326 (B-E) and MK2 phosphorylated at T334
824 (D) were detected by western blot analysis. The levels of α -tubulin (A) or β -actin (B-
825 E) served as loading controls.

826

827 **Figure 8. S326A/E mutation does not influence the nucleo-cytoplasmic**
828 **distribution of ectopically expressed HSF1-GFP.** HSF1-knockout MEFs were
829 transfected with GFP-tagged wild-type or S326A/E mutants of HSF1 prior to counter
830 staining with anti-ERK1/2 antibodies and DAPI. Four fields of view per well of eight
831 replicate wells per condition were imaged using a robotic high content microscope.
832 Automated and systematic analysis of images was performed using a custom
833 algorithm. **(A)** A single representative field of view is shown from one well. DAPI
834 and ERK1/2 images were used to define nuclear and cytoplasmic regions,
835 respectively, and GFP fluorescence was recorded from each region (as indicated in
836 the inset screengrab showing automated cell definition), accepting >70 AFU per cell
837 as positively transfected. **(B)** Plots of single cell data show a frequency histogram of
838 nuclear : cytoplasmic GFP fluorescence (left panel) indicating a bimodal distribution
839 of HSF1, which is unaffected by S326A/E mutation. The right hand panel shows a
840 comparison of whole cell GFP fluorescence in the same cell populations versus
841 nucleo-cytoplasmic distribution, indicating that the bimodal distribution is apparent
842 across a 10-fold difference in HSF1-GFP levels, and is again unaffected by S326A/E
843 substitution.

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REFERENCES

1. **Morimoto RI.** 2011. The heat shock response: systems biology of proteotoxic stress in aging and disease. *Cold Spring Harb Symp Quant Biol* **76**:91-99.
2. **Anckar J, Sistonen L.** 2011. Regulation of HSF1 function in the heat stress response: implications in aging and disease. *Annu Rev Biochem* **80**:1089-1115.
3. **Vihervaara A, Sistonen L.** 2014. HSF1 at a glance. *J Cell Sci* **127**:261-266.
4. **Richter K, Haslbeck M, Buchner J.** 2010. The heat shock response: life on the verge of death. *Mol Cell* **40**:253-266.
5. **Mendillo ML, Santagata S, Koeva M, Bell GW, Hu R, Tamimi RM, Fraenkel E, Ince TA, Whitesell L, Lindquist S.** 2012. HSF1 drives a transcriptional program distinct from heat shock to support highly malignant human cancers. *Cell* **150**:549-562.
6. **Dai C, Santagata S, Tang Z, Shi J, Cao J, Kwon H, Bronson RT, Whitesell L, Lindquist S.** 2012. Loss of tumor suppressor NF1 activates HSF1 to promote carcinogenesis. *J Clin Invest* **122**:3742-3754.
7. **Calderwood SK.** 2012. HSF1, a versatile factor in tumorigenesis. *Curr Mol Med* **12**:1102-1107.
8. **Gabai VL, Meng L, Kim G, Mills TA, Benjamin IJ, Sherman MY.** 2012. Heat shock transcription factor Hsf1 is involved in tumor progression via regulation of hypoxia-inducible factor 1 and RNA-binding protein HuR. *Mol Cell Biol* **32**:929-940.
9. **Chou SD, Murshid A, Eguchi T, Gong J, Calderwood SK.** 2014. HSF1 regulation of beta-catenin in mammary cancer cells through control of HuR/elavL1 expression. *Oncogene* **34**:2178-88.
10. **Santagata S, Mendillo ML, Tang YC, Subramanian A, Perley CC, Roche SP, Wong B, Narayan R, Kwon H, Koeva M, Amon A, Golub TR, Porco JA, Jr., Whitesell L, Lindquist S.** 2013. Tight coordination of protein translation and HSF1 activation supports the anabolic malignant state. *Science* **341**:1238303.
11. **Westerheide SD, Anckar J, Stevens SM, Jr., Sistonen L, Morimoto RI.** 2009. Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. *Science* **323**:1063-1066.
12. **Raychaudhuri S, Loew C, Korner R, Pinkert S, Theis M, Hayer-Hartl M, Buchholz F, Hartl FU.** Interplay of acetyltransferase EP300 and the proteasome system in regulating heat shock transcription factor 1. *Cell* **156**:975-985.
13. **Budzynski MA, Puustinen MC, Joutsen J, Sistonen L.** 2015. Uncoupling stress-inducible phosphorylation of heat shock factor 1 from its activation. *Mol Cell Biol* **35**:2530-2540.
14. **Kline MP, Morimoto RI.** 1997. Repression of the heat shock factor 1 transcriptional activation domain is modulated by constitutive phosphorylation. *Mol Cell Biol* **17**:2107-2115.
15. **Chu B, Zhong R, Soncin F, Stevenson MA, Calderwood SK.** 1998. Transcriptional activity of heat shock factor 1 at 37 degrees C is repressed through phosphorylation on two distinct serine residues by glycogen synthase kinase 3 and protein kinases Calpha and Czeta. *J Biol Chem* **273**:18640-18646.
16. **Xavier IJ, Mercier PA, McLoughlin CM, Ali A, Woodgett JR, Ovsenek N.** 2000. Glycogen synthase kinase 3 β negatively regulates both DNA-binding

- 899 and transcriptional activities of heat shock factor 1. *J Biol Chem* **275**:29147-
900 29152.
- 901 17. **Knauf U, Newton EM, Kyriakis J, Kingston RE.** 1996. Repression of
902 human heat shock factor 1 activity at control temperature by phosphorylation.
903 *Genes Dev* **10**:2782-2793.
- 904 18. **Wang X, Khaleque MA, Zhao MJ, Zhong R, Gaestel M, Calderwood SK.**
905 2006. Phosphorylation of HSF1 by MAPK-activated protein kinase 2 on serine
906 121, inhibits transcriptional activity and promotes HSP90 binding. *J Biol*
907 *Chem* **281**:782-791.
- 908 19. **Guettoche T, Boellmann F, Lane WS, Voellmy R.** 2005. Analysis of
909 phosphorylation of human heat shock factor 1 in cells experiencing a stress.
910 *BMC Biochem* **6**:4.
- 911 20. **Chou SD, Prince T, Gong J, Calderwood SK.** 2012. mTOR is essential for
912 the proteotoxic stress response, HSF1 activation and heat shock protein
913 synthesis. *PLoS One* **7**:e39679.
- 914 21. **Kristal AR, Lampe JW.** 2002. Brassica vegetables and prostate cancer risk: a
915 review of the epidemiological evidence. *Nutr Cancer* **42**:1-9.
- 916 22. **Fahey JW, Zalcman AT, Talalay P.** 2001. The chemical diversity and
917 distribution of glucosinolates and isothiocyanates among plants.
918 *Phytochemistry* **56**:5-51.
- 919 23. **Mithen R, Bennett R, Marquez J.** 2010. Glucosinolate biochemical diversity
920 and innovation in the Brassicales. *Phytochemistry* **71**:2074-2086.
- 921 24. **Hu R, Xu C, Shen G, Jain MR, Khor TO, Gopalkrishnan A, Lin W,**
922 **Reddy B, Chan JY, Kong AN.** 2006. Identification of Nrf2-regulated genes
923 induced by chemopreventive isothiocyanate PEITC by oligonucleotide
924 microarray. *Life Sci* **79**:1944-1955.
- 925 25. **Cheung KL, Khor TO, Yu S, Kong AN.** 2008. PEITC induces G1 cell cycle
926 arrest on HT-29 cells through the activation of p38 MAPK signaling pathway.
927 *Aaps J* **10**:277-281.
- 928 26. **Zhang Y, Ahn YH, Benjamin IJ, Honda T, Hicks RJ, Calabrese V, Cole**
929 **PA, Dinkova-Kostova AT.** 2011. HSF1-dependent upregulation of Hsp70 by
930 sulfhydryl-reactive inducers of the KEAP1/NRF2/ARE pathway. *Chem Biol*
931 **18**:1355-1361.
- 932 27. **Dinkova-Kostova AT.** 2012. Chemoprotection against cancer by
933 isothiocyanates: a focus on the animal models and the protective mechanisms.
934 *Top Curr Chem* **329**:179-201.
- 935 28. **Dinkova-Kostova AT, Kostov RV.** 2012. Glucosinolates and isothiocyanates
936 in health and disease. *Trends Mol Med* **18**:337-347.
- 937 29. **Kensler TW, Egner PA, Agyeman AS, Visvanathan K, Groopman JD,**
938 **Chen JG, Chen TY, Fahey JW, Talalay P.** 2012. Keap1-Nrf2 signaling: a
939 target for cancer prevention by sulforaphane. *Top Curr Chem* **329**:163-177.
- 940 30. **Sorger PK, Nelson HC.** 1989. Trimerization of a yeast transcriptional
941 activator via a coiled-coil motif. *Cell* **59**:807-813.
- 942 31. **Perisic O, Xiao H, Lis JT.** 1989. Stable binding of Drosophila heat shock
943 factor to head-to-head and tail-to-tail repeats of a conserved 5 bp recognition
944 unit. *Cell* **59**:797-806.
- 945 32. **Peteranderl R, Nelson HC.** 1992. Trimerization of the heat shock
946 transcription factor by a triple-stranded alpha-helical coiled-coil. *Biochemistry*
947 **31**:12272-12276.

- 948 33. **Rabindran SK, Haroun RI, Clos J, Wisniewski J, Wu C.** 1993. Regulation
949 of heat shock factor trimer formation: role of a conserved leucine zipper.
950 *Science* **259**:230-234.
- 951 34. **Calamini B, Silva MC, Madoux F, Hutt DM, Khanna S, Chalfant MA,**
952 **Saldanha SA, Hodder P, Tait BD, Garza D, Balch WE, Morimoto RI.**
953 2012. Small-molecule proteostasis regulators for protein conformational
954 diseases. *Nat Chem Biol* **8**:185-196.
- 955 35. **Jhaveri K, Taldone T, Modi S, Chiosis G.** 2012. Advances in the clinical
956 development of heat shock protein 90 (Hsp90) inhibitors in cancers. *Biochim*
957 *Biophys Acta* **1823**:742-755.
- 958 36. **Taipale M, Krykbaeva I, Koeva M, Kayatekin C, Westover KD, Karras**
959 **GI, Lindquist S.** 2012. Quantitative analysis of HSP90-client interactions
960 reveals principles of substrate recognition. *Cell* **150**:987-1001.
- 961 37. **Zhang Y, Dayalan Naidu S, Samarasinghe K, Van Hecke GC, Pheely A,**
962 **Boronina TN, Cole RN, Benjamin IJ, Cole PA, Ahn YH, Dinkova-**
963 **Kostova AT.** 2014. Sulphoxythiocarbamates modify cysteine residues in
964 HSP90 causing degradation of client proteins and inhibition of cancer cell
965 proliferation. *Br J Cancer* **110**:71-82.
- 966 38. **Ubersax JA, Ferrell JE, Jr.** 2007. Mechanisms of specificity in protein
967 phosphorylation. *Nat Rev Mol Cell Biol* **8**:530-541.
- 968 39. **Park J, Liu AY.** 2001. JNK phosphorylates the HSF1 transcriptional
969 activation domain: role of JNK in the regulation of the heat shock response. *J*
970 *Cell Biochem* **82**:326-338.
- 971 40. **Zhang T, Inesta-Vaquera F, Niepel M, Zhang J, Ficarro SB, Machleidt T,**
972 **Xie T, Marto JA, Kim N, Sim T, Laughlin JD, Park H, LoGrasso PV,**
973 **Patricelli M, Nomanbhoy TK, Sorger PK, Alessi DR, Gray NS.** 2012.
974 Discovery of potent and selective covalent inhibitors of JNK. *Chem Biol*
975 **19**:140-154.
- 976 41. **Kuma Y, Sabio G, Bain J, Shpiro N, Marquez R, Cuenda A.** 2005.
977 BIRB796 inhibits all p38 MAPK isoforms in vitro and in vivo. *J Biol Chem*
978 **280**:19472-19479.
- 979 42. **Tang Z, Dai S, He Y, Doty RA, Shultz LD, Sampson SB, Dai C.** 2015.
980 MEK guards proteome stability and inhibits tumor-suppressive
981 amyloidogenesis via HSF1. *Cell* **160**:729-744.
- 982 43. **Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser**
983 **WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda**
984 **RL, Scherle PA, Trzaskos JM.** 1998. Identification of a novel inhibitor of
985 mitogen-activated protein kinase kinase. *J Biol Chem* **273**:18623-18632.
- 986 44. **Goedert M, Cuenda A, Craxton M, Jakes R, Cohen P.** 1997. Activation of
987 the novel stress-activated protein kinase SAPK4 by cytokines and cellular
988 stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity
989 with that of other SAP kinases. *EMBO J* **16**:3563-3571.
- 990 45. **Zur R, Garcia-Ibanez L, Nunez-Buiza A, Aparicio N, Liappas G, Escos A,**
991 **Risco A, Page A, Saiz-Ladera C, Alsina-Beauchamp D, Montans J,**
992 **Paramio JM, Cuenda A.** 2015. Combined deletion of p38 γ and p38 δ reduces
993 skin inflammation and protects from carcinogenesis. *Oncotarget* **6**:12920-
994 12935.
- 995 46. **Morley JF, Morimoto RI.** 2004. Regulation of longevity in *Caenorhabditis*
996 *elegans* by heat shock factor and molecular chaperones. *Mol Biol Cell* **15**:657-
997 664.

- 998 47. **Chiang WC, Ching TT, Lee HC, Mousigian C, Hsu AL.** 2012. HSF-1
999 regulators DDL-1/2 link insulin-like signaling to heat-shock responses and
1000 modulation of longevity. *Cell* **148**:322-334.
- 1001 48. **Xu YM, Huang DY, Chiu JF, Lau AT.** 2012. Post-translational modification
1002 of human heat shock factors and their functions: a recent update by proteomic
1003 approach. *J Proteome Res* **11**:2625-2634.
- 1004 49. **Soncin F, Zhang X, Chu B, Wang X, Asea A, Ann Stevenson M, Sacks DB,**
1005 **Calderwood SK.** 2003. Transcriptional activity and DNA binding of heat
1006 shock factor-1 involve phosphorylation on threonine 142 by CK2. *Biochem*
1007 *Biophys Res Commun* **303**:700-706.
- 1008 50. **Lee YJ, Kim EH, Lee JS, Jeoung D, Bae S, Kwon SH, Lee YS.** 2008. HSF1
1009 as a mitotic regulator: phosphorylation of HSF1 by Plk1 is essential for
1010 mitotic progression. *Cancer Res* **68**:7550-7560.
- 1011 51. **Kim SA, Yoon JH, Lee SH, Ahn SG.** 2005. Polo-like kinase 1
1012 phosphorylates heat shock transcription factor 1 and mediates its nuclear
1013 translocation during heat stress. *J Biol Chem* **280**:12653-12657.
- 1014 52. **Murshid A, Chou SD, Prince T, Zhang Y, Bharti A, Calderwood SK.** 2010.
1015 Protein kinase A binds and activates heat shock factor 1. *PLoS One* **5**:e13830.
- 1016 53. **Holmberg CI, Hietakangas V, Mikhailov A, Rantanen JO, Kallio M,**
1017 **Meinander A, Hellman J, Morrice N, MacKintosh C, Morimoto RI,**
1018 **Eriksson JE, Sistonen L.** 2001. Phosphorylation of serine 230 promotes
1019 inducible transcriptional activity of heat shock factor 1. *EMBO J* **20**:3800-
1020 3810.
- 1021 54. **Cavell BE, Syed Alwi SS, Donlevy AM, Proud CG, Packham G.** 2012.
1022 Natural product-derived antitumor compound phenethyl isothiocyanate
1023 inhibits mTORC1 activity via TSC2. *J Nat Prod* **75**:1051-1057.
- 1024 55. **Cohen P.** 2009. Targeting protein kinases for the development of anti-
1025 inflammatory drugs. *Curr Opin Cell Biol* **21**:317-324.
- 1026 56. **Banerjee Mustafi S, Chakraborty PK, Dey RS, Raha S.** 2009. Heat stress
1027 upregulates chaperone heat shock protein 70 and antioxidant manganese
1028 superoxide dismutase through reactive oxygen species (ROS), p38MAPK, and
1029 Akt. *Cell Stress Chaperones* **14**:579-589.
- 1030 57. **Sugimoto N, Shido O, Matsuzaki K, Ohno-Shosaku T, Hitomi Y, Tanaka**
1031 **M, Sawaki T, Fujita Y, Kawanami T, Masaki Y, Okazaki T, Nakamura H,**
1032 **Koizumi S, Yachie A, Umehara H.** 2012. Cellular heat acclimation regulates
1033 cell growth, cell morphology, mitogen-activated protein kinase activation, and
1034 expression of aquaporins in mouse fibroblast cells. *Cell Physiol Biochem*
1035 **30**:450-457.
- 1036 58. **Seo MS, Oh SY, Park MJ, Kim SM, Kim MY, Han SI, Park HG, Kang**
1037 **HS.** 2005. Implication of reactive oxygen species, ERK1/2, and p38MAPK in
1038 sodium salicylate-induced heat shock protein 72 expression in C6 glioma cells.
1039 *Int J Mol Med* **16**:841-849.
- 1040 59. **Westerheide SD, Bosman JD, Mbadugha BN, Kawahara TL, Matsumoto**
1041 **G, Kim S, Gu W, Devlin JP, Silverman RB, Morimoto RI.** 2004. Celastrols
1042 as inducers of the heat shock response and cytoprotection. *J Biol Chem*
1043 **279**:56053-56060.
- 1044 60. **Dayalan Naidu S, Kostov RV, Dinkova-Kostova AT.** 2015. Transcription
1045 factors Hsf1 and Nrf2 engage in crosstalk for cytoprotection. *Trends*
1046 *Pharmacol Sci* **36**:6-14.

- 1047 61. **Trott A, West JD, Klaic L, Westerheide SD, Silverman RB, Morimoto RI,**
1048 **Morano KA.** 2008. Activation of heat shock and antioxidant responses by the
1049 natural product celastrol: transcriptional signatures of a thiol-targeted
1050 molecule. *Mol Biol Cell* **19**:1104-1112.
- 1051 62. **Escos A, Risco A, Alsina-Beauchamp D, Cuenda A.** 2016. p38 γ and p38 δ
1052 mitogen activated protein kinases (MAPKs), new stars in the MAPK galaxy.
1053 *Front Cell Dev Biol* **4**:31.
- 1054 63. **Xiao X, Zuo X, Davis AA, McMillan DR, Curry BB, Richardson JA,**
1055 **Benjamin IJ.** 1999. HSF1 is required for extra-embryonic development,
1056 postnatal growth and protection during inflammatory responses in mice.
1057 *EMBO J* **18**:5943-5952.
- 1058 64. **Suzuki K, Bose P, Leong-Quong RY, Fujita DJ, Riabowol K.** 2010. REAP:
1059 A two minute cell fractionation method. *BMC Res Notes* **3**:294.
1060

Fig. 1

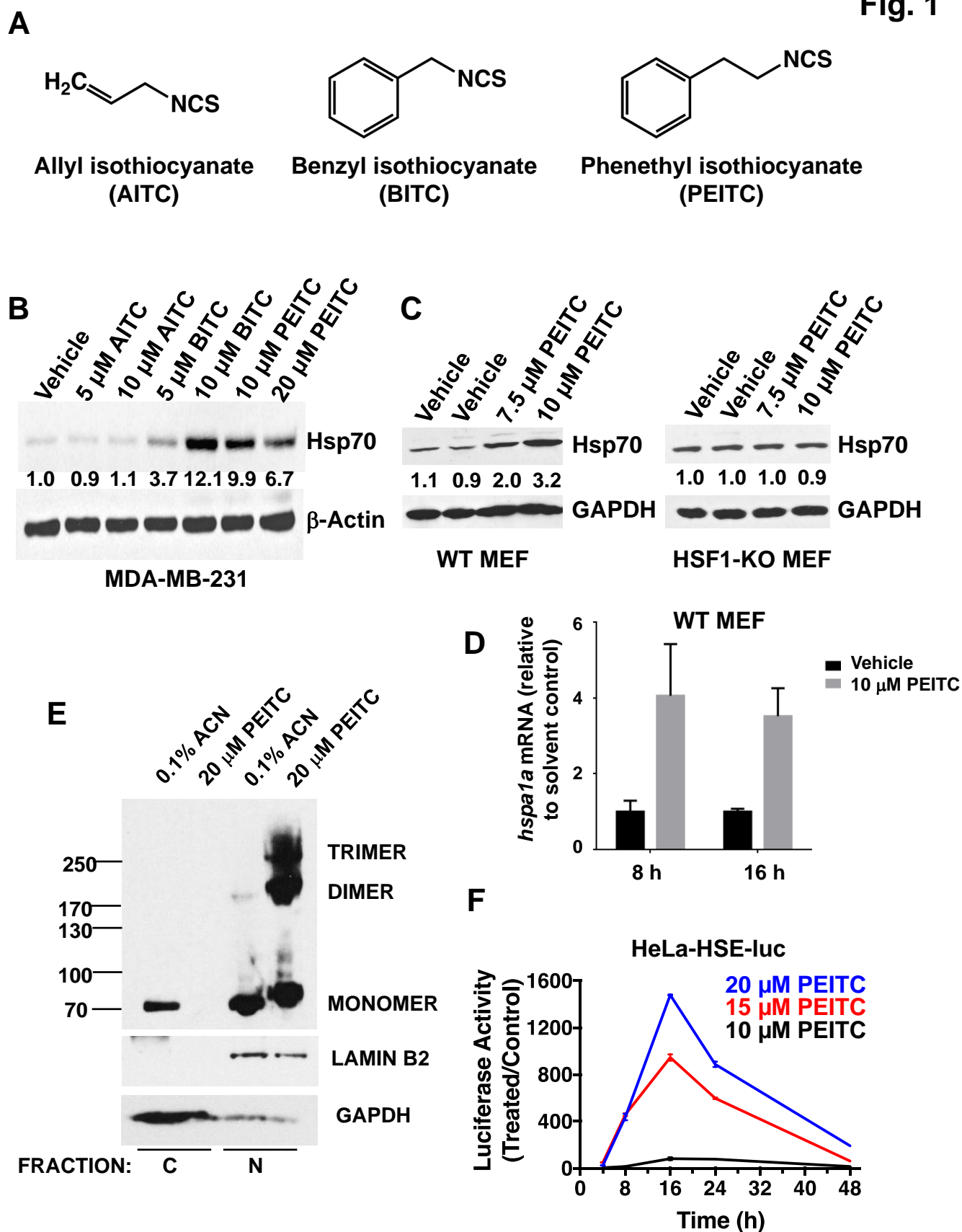


Fig. 2

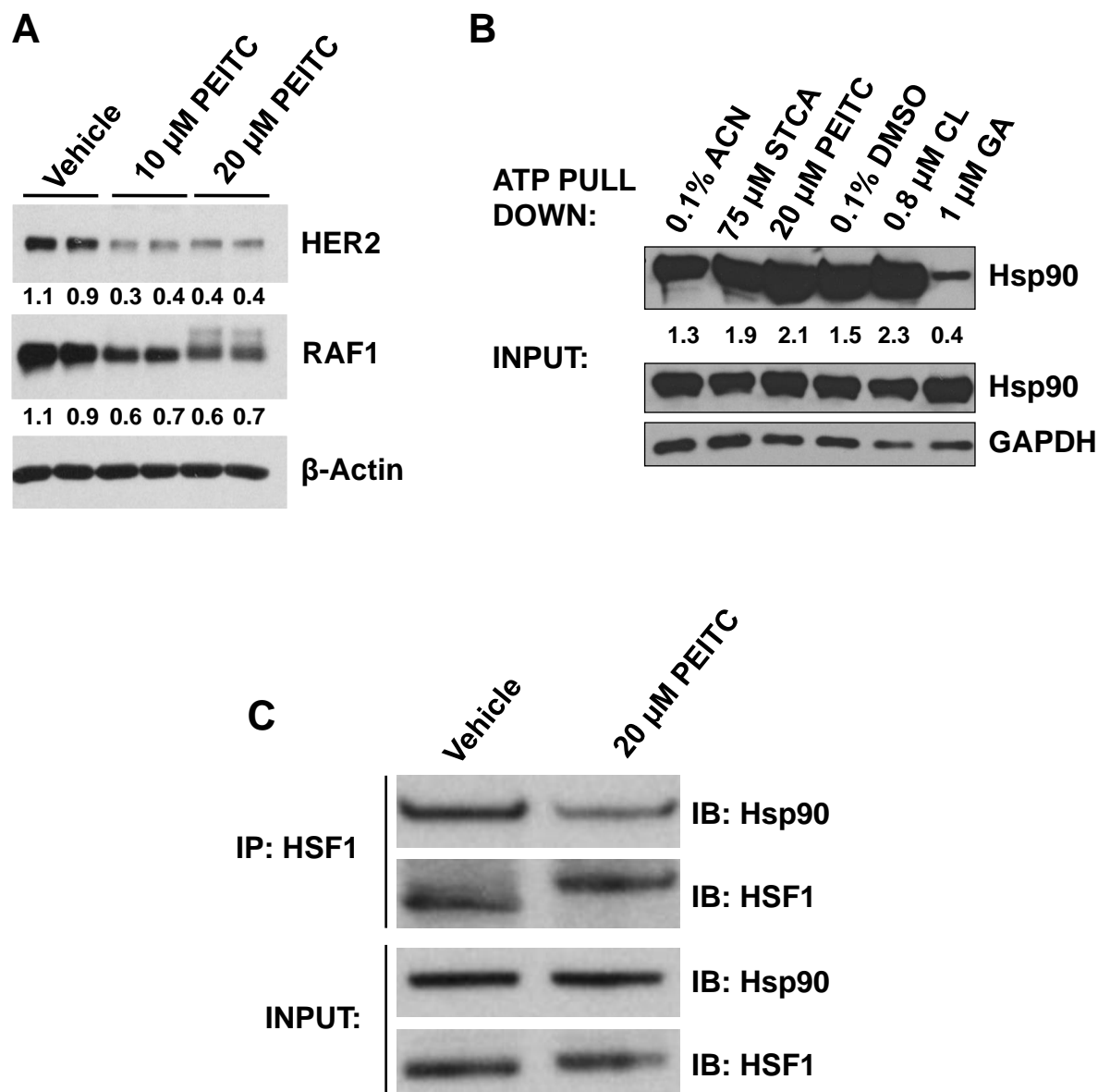


Fig. 3

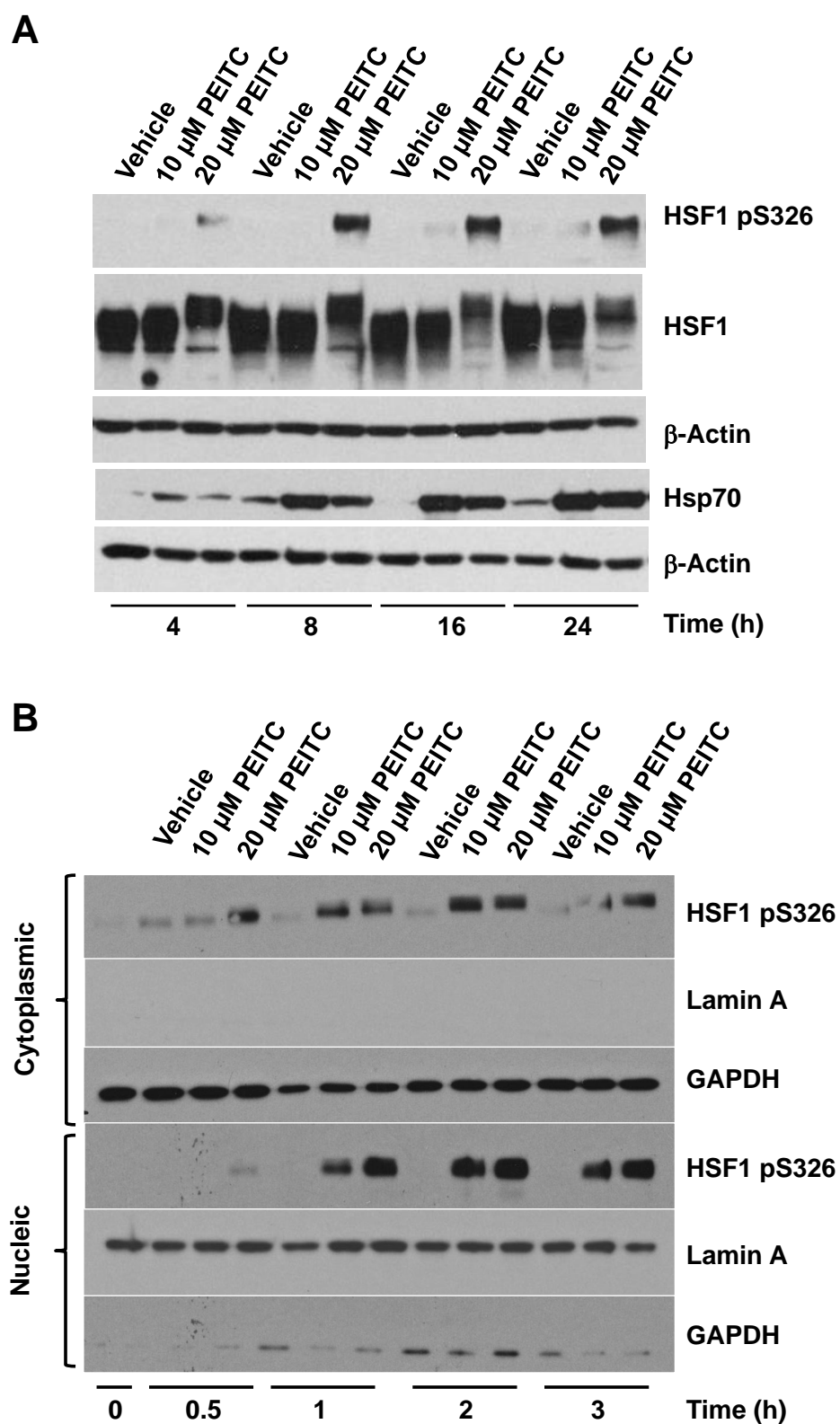


Fig. 4

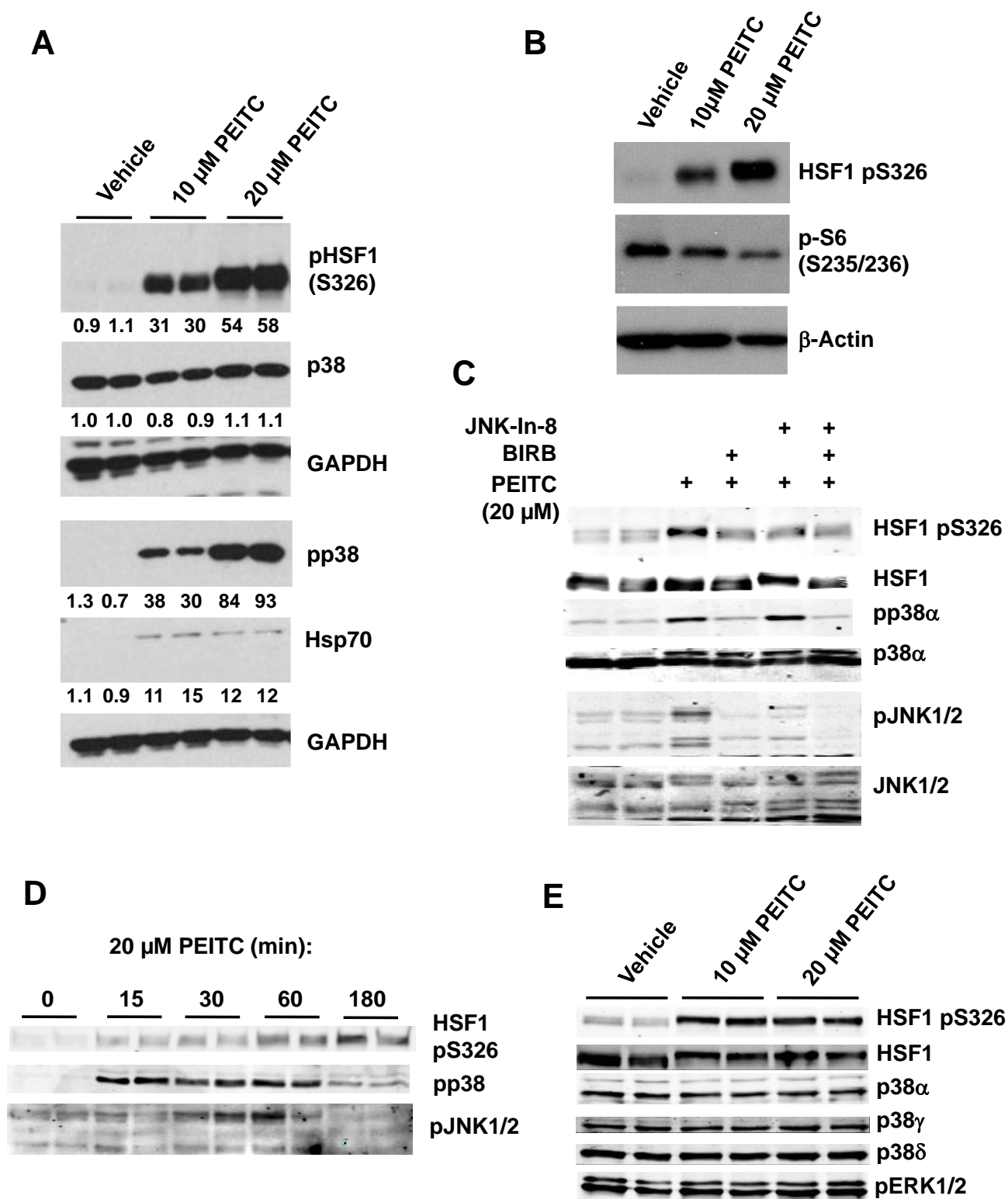
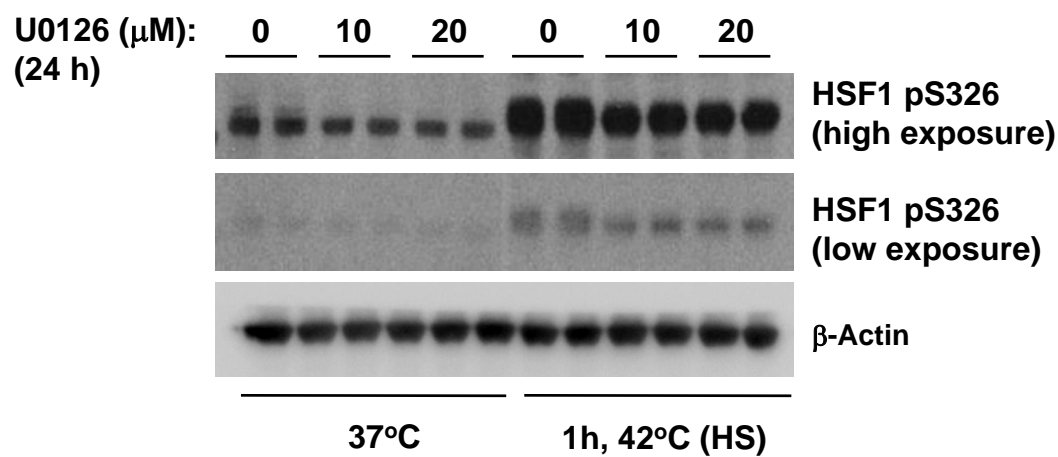


Fig. 5

A



B

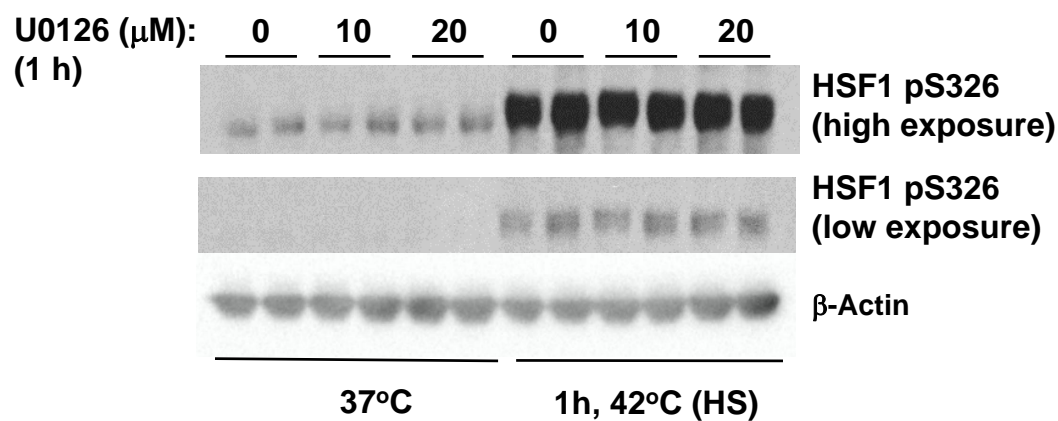


Fig. 6

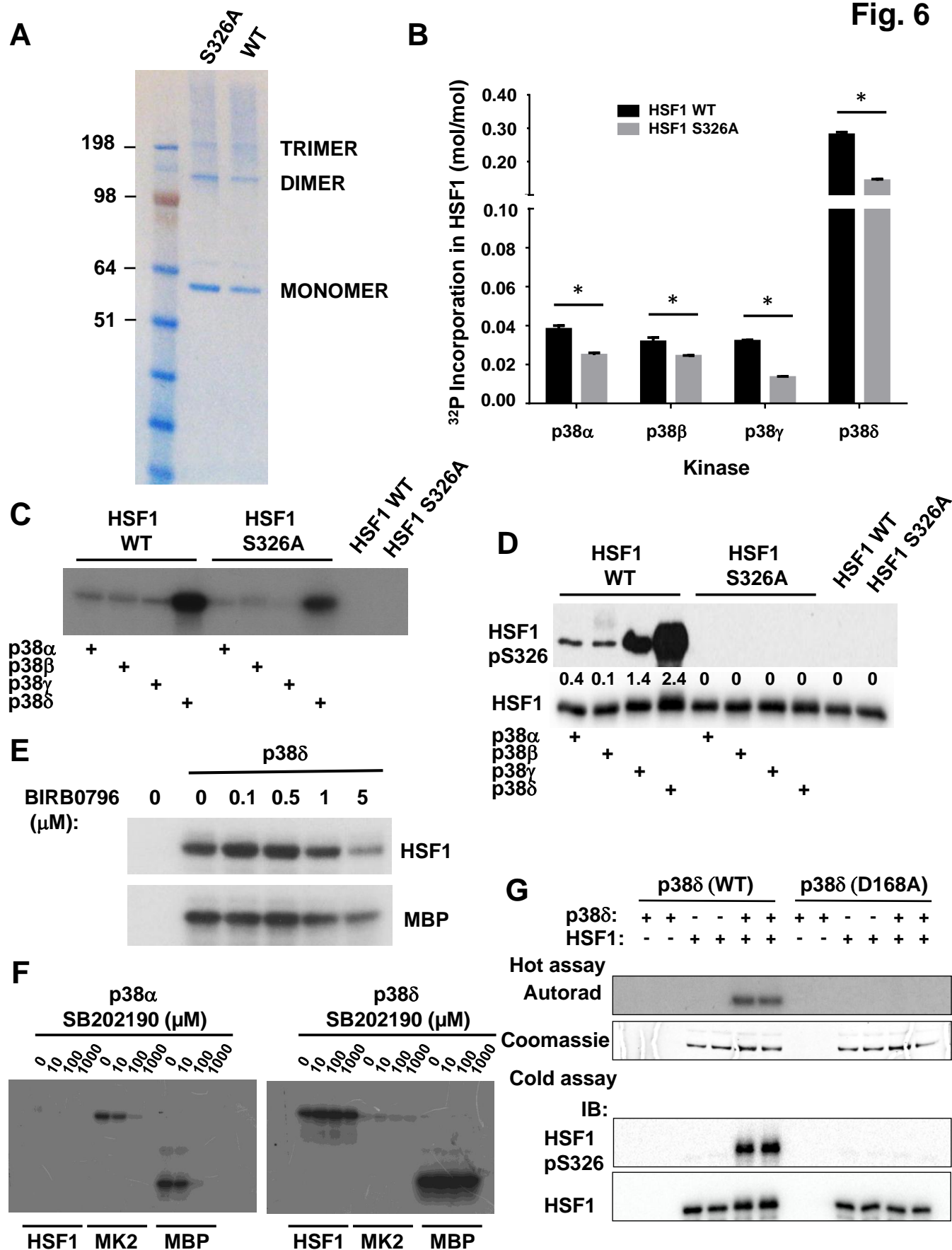


Fig. 7

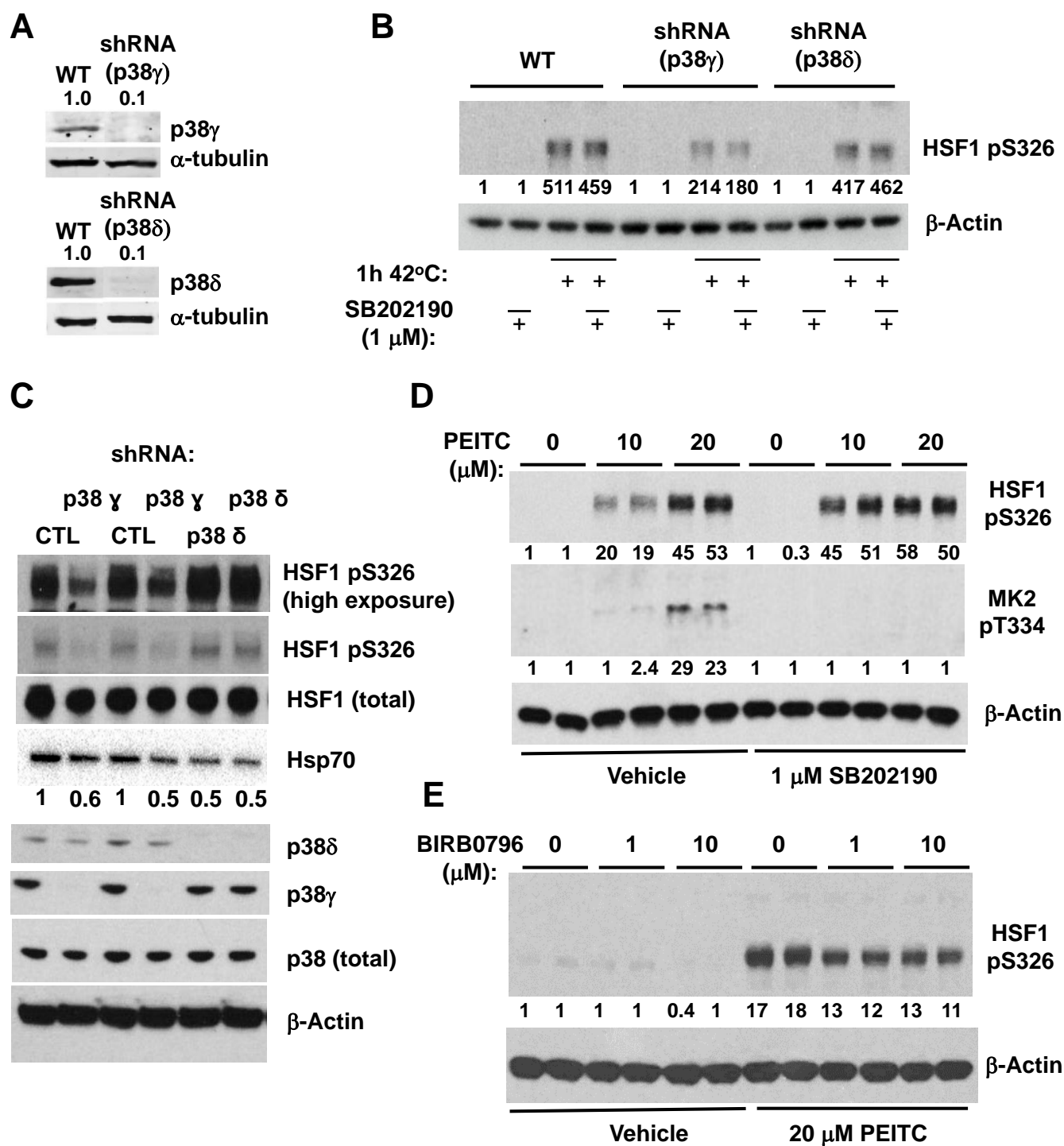


Fig. 8

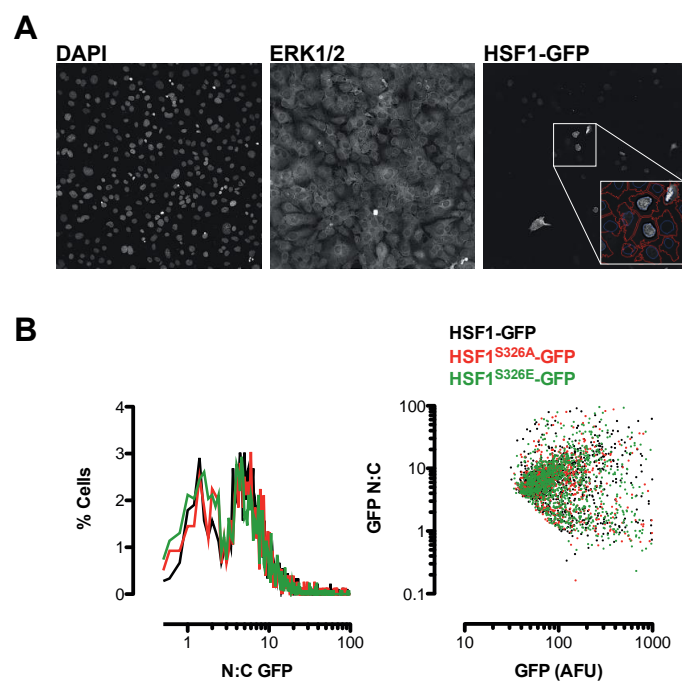


Table 1. Phosphopeptides identified by liquid chromatography tandem mass spectrometry after 60-min incubation of recombinant human HSF1 (1.0 μ g) with p38 MAPK α or p38 MAPK δ (0.06 mU/ μ l), followed by SDS/PAGE separation, and in-gel tryptic digestion.

p38 MAPKα + HSF1 Peptide sequence	Phospho-S	M (calc.)	M (expt.)
K.EEPPS ³⁰³ PPQS ³⁰⁷ PR.V	S303/307	1299.5496	1299.5487
R.VKEEPPS ³⁰³ PPQS ³⁰⁷ PR.V	S303/307	1526.7130	1526.7121
R.VEEASPGRPSSVDTLLS ³²⁶ PTALIDSILR.E	S326	2902.4689	2902.4659
p38 MAPKδ + HSF1 Peptide sequence	Phospho-S	M (calc.)	M (expt.)
K.EEPPS ³⁰³ PPQS ³⁰⁷ PR.V	S303/307	1299.5496	1299.5493
R.VKEEPPS ³⁰³ PPQS ³⁰⁷ PR.V	S303/307	1526.7130	1526.7121
R.VEEASPGRPSSVDTLLS ³²⁶ PTALIDSILR.E	S326	2902.4689	2902.4683